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Metabolites of Two Fungi:
Penicillium brevi-compactum (Dierckx)
and *Gremmeniella abietina* (Lagerb.)

by

Ⓒ Ian Andrew van Altena

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Metabolites of Two Fungi: Penicillium brevi-compactum (Dierckx) and Gremmeniella abietina (Lagerb.) submitted by Ian Andrew van Altena in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

. . . .
Supervisor--W.A. Ayer

. . . .

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H B Dunford
. . . .

D. L. J. Clive
. . . .

Albert Stoessl
. . . .
External Examiner

Date. 23rd June 1982

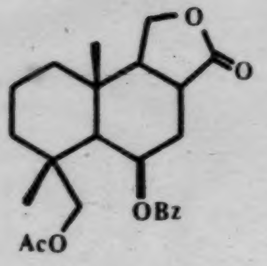
When I consider Thy heavens, the work of Thy fingers,
The moon and the stars, which Thou hast ordained;
What is man, that Thou dost take thought of him?
And the son of man, that Thou dost care for him?
Yet Thou hast made him a little lower than the angels,
And dost crown him with glory and majesty!
Thou dost make him to rule over the works of Thy hands;
Thou hast put all things under his feet.

Psalms 8:3-6

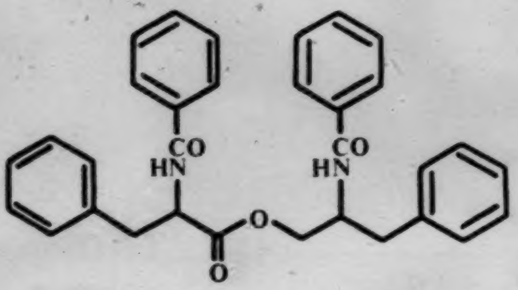
Abstract

The metabolites of two fungi, *Penicillium brevi-compactum* an antagonist of Dutch Elm disease fungus *Ceratocystis ulmi*; and *Gremmeniella abietina*, a pathogen of pine trees, were investigated.

P. brevi-compactum was grown in still and fermentation culture. Two known compounds, 1-deoxypebrolide (I) and asperphenamate (II), were isolated from the mycelium of the still culture.

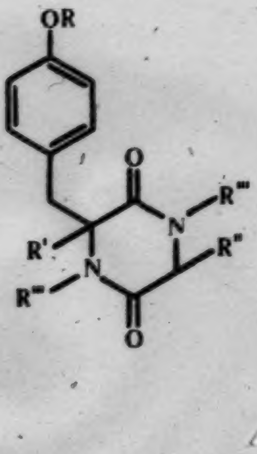


I.



II.

A series of piperazine-2,5-dione compounds (III-VII) which were identified by spectroscopic methods and by comparison to synthetic



III. R=H; R',R"=SCH_3; R'''=CH_3.

IV. R=$CH_2CHC(CH_3)_2$; R',R"=SCH_3; R'''=CH_3.

V. R=$CH_2CHC(CH_3)_2$; R'=SCH_3; R",R'''=H.

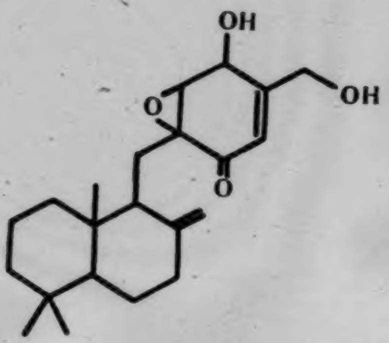
VI. R=$CH_2CHC(CH_3)_2$; R'=SCH_3; R"=OH; R'''=H.

VII. R,R'''=CH_3; R'=OH; R"=H.

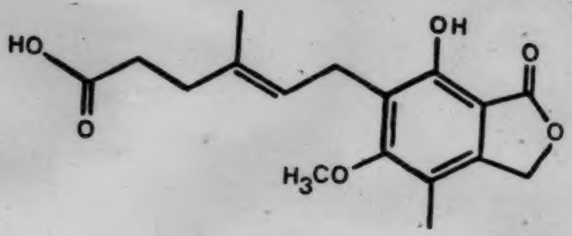
VIII. R,R'''=CH_3; R',R"=H.

model compound 8 were present in the broth of both still and fermentation cultures.

Compound 9, for which we propose structure 9 based on spectroscopic evidence and chemical transformations, was isolated from an early fermentation of P. brevi-compactum.



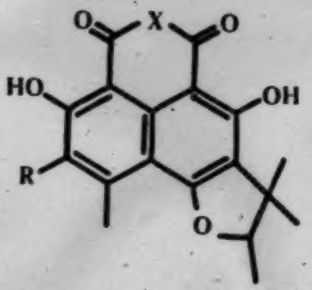
IX.



X.

Mycophenolic acid (X), a major metabolite of this fungus was isolated from all cultures investigated.

When G. abietina was grown by fermentation a series of related naphthalene-1,8-dicarboxylic acid anhydride (XI, XII) and phenalenone (XIII) compounds was isolated. One metabolite, the purple crystalline trione 12, is the probable cause of the green-blue colouration of the tissues of pine trees infected by G. abietina.



XI. R=H; x=O
XII. R=OH; x=O
XIII. R=H; x=CO

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Introduction

The work described in this thesis forms part of the continuing work being carried out in these laboratories on the metabolites of biologically interesting fungi.

In recent times, fungi have become the concern of not only the chemist but also the taxonomist, morphologist, geneticist, ecologist, plant pathologist, physician, biochemist, and commercial microbiologist, among others. This list reflects the ubiquity of the fungi, their usefulness as research organisms, and their involvement in many facets of our everyday lives[1]. Some familiar examples of fungi in everday life are mushrooms, puff balls, yeast (bread and beer), cheese, diseases such as corn smut, black stem rust of wheat, athlete's foot, ring worm, and as a source of drugs-antibiotics and hallucinogens.

Penicillium brevi-compactum, a fungus antagonistic towards the Dutch Elm disease fungus Ceratocystis ulmi, and Gremmeniella abietina, a virulent pathogen of red and jack pine in North America[2], were obtained from Dr. Y. Hiratsuka of the Northern Forest Research Centre, Edmonton.

Our interest in these fungi is based on the fact that forests constitute one of our most important natural resources, contributing wood for innumerable uses by man. Deterioration of wood is caused by such things as abrasion, termites, and marine borers, but fungi cause the most serious problems. Of the types of materials affected by fungi, the greatest amount of damage occurs to wood in the form of standing timber, rough lumber, or finished products including paper.

Fungi may attack the heartwood of standing living trees making it unfit for lumber and often leading to the early death of the tree through structural weakening. Fungi acting as parasites can impair water and mineral absorption due to root damage, through generalised disintegration of the tissue through which water must pass, and through gum formation in the xylem region.

Dutch Elm disease is one such disease of the vascular system, causing local necrosis in the xylem which in turn causes the tree to produce gels and gums to defend against the fungus. However, these are only partially effective in limiting the upward growth of the fungus and flow of toxins. Desication systems are primarily due to toxic metabolites transported to the leaves. The leaves are particularly vulnerable as leaf cells have a greater permeability and an impaired ability to regulate their osmotic functions resulting in a loss of turgor and increased transpiration[3].

The fungus[4] which causes Dutch Elm disease (_C. ulmi_) was first discovered in Canada (Richelieu County) in 1944 having been imported from Europe. It has since spread throughout Canada as far as Manitoba, from where its spread to the north and west is restricted due to the sparse population of elm trees.

The fungus is spread by bark beetles which carry fungal spores from the dead trees to living ones. Small trees, once infected, can die in a single season whereas larger trees can survive for a number of years.

Control of the disease is at best quarantine in nature _viz._ burning of infected trees and spraying uninfected trees with insecticide in order to kill the bark beetles before they transmit the fungal spores. Treatment of infected trees is both expensive and not very effective at this point in time: e.g., seven out of twenty badly diseased trees can be saved by injecting pseudomonad bacteria which attack C. ulmi. An alternative approach has been to breed resistant trees but mutation and the commercial fact of life that these trees don't sell has made progress in this area unrewarding[5]*.

A desirable method of control of C. ulmi is a chemical one where an antifungal agent is applied to the tree either in a preventative or therapeutic sense. The discovery that our strain of Penicillium species was antagonistic toward C. ulmi[6] led us to investigate its metabolites in search of its active component(s).

A survey of the chemical literature indicated that P. brevi-compactum has been studied by natural products chemists over a considerable time span beginning with Clutterbuck and Raistrick[7] in 1932. Among the compounds identified including various phenols, lipids, and steroids, a number of physiologically or structurally interesting molecules have been reported.

Perhaps the most outstanding of these is mycophenolic acid (1) isolated by Raistrick and Clutterbuck[7]. It shows, for example, some antibiotic activity[8, 9], high activity as an antifungal agent[10], phytotoxicity and/or instability in aqueous solutions[11], and activity as an antineoplastic agent[12, 13]. It is currently being developed in therapeutic applications.

---

*For a very complete review of the Dutch Elm disease problem see Dutch Elm Disease: Perspectives After 60 Years in Search, Agriculture Volume 8, No. 5 Plant Pathology 1 (1978) ed. W.A. Sinclaire, R.J. Campana A Northeast Research Publication (U.S.A.).

A more readable and up to date review can be found in Scientific American 245, 56 (1981) by G.A. Strobel, G.N. Lanier.

In 1966, McCorkindale et al.[14] reported three compounds whose structures were related closely to mycophenolic acid. This group later reported the existence of three further metabolites, pebrolide (2)[15], and two related sesquiterpene benzoates[16], desacetyl pebrolide and 1-deoxypebrolide.

In 1971, Birch reported the structure of brevianamide A (3)[17], a polar compound from P. brevi-compactum. This was later joined by brevianamides B, C (4), D, and F (5)[18]. In the same year Law and co-workers reported[19] the isolation of several ubiquinone homologues (6) and ubichromenol-8 from P. brevi-compactum

Lastly, compactin (7) was isolated by Brown et al. and was shown to have antifungal activity[20] and later, to be extremely potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an important enzyme in the cholesterol biosynthetic pathway[21].

In contrast to P. brevi-compactum, Gremmeniella abietina (lagerb.) seems to be unstudied as far as the chemical literature is concerned. G. abietina, a pathogen of at least ten species of European conifers causing defoliation, dieback and/or cankers, was first noticed in Canada in 1951. In Canada it has been observed to infect red pine (Pinus resinosa), killing tops and lateral branches of three foot or smaller trees and lower branches in larger trees, as well as jack pine, (P. banksiana) killing entire trees through girdling, elongated cankers that extend above the ground line for ten to fifteen centimeters.

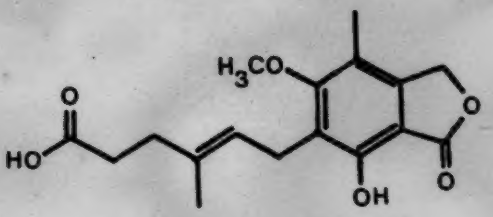
An interesting symptom of G. abietina infection is a characteristic yellow-green discolouration in the cambrial zones of recently killed red and jack pine tissues. Sometimes a green margin is observed in these tissues. In trees that have been dead for a year or more the colour has changed to greenish-blue. The relationship between these colours and the fungus itself was established early on in our work.

Lastly, it is worth mentioning that this fungus is somewhat unique in the conditions it requires for growth in nature: the temperature it needs for best growth is in the range of 13 to 20° C, significantly lower than normal. This fact explains why trees in areas prone to summer frost were attacked more vigourously and also explains the initial problems encountered in the actual isolation of the fungus from infected trees[2, 6].

As chemists we are interested in the identity and properties of the metabolites in these fungi, particularly any metabolites that exhibit biological activity. This involves establishing methods to grow the fungi, assaying for bioactivity, separating the metabolites from the natural substrate and from each other using established chemical and physical techniques, identifying the metabolites, and suggesting practical applications of the knowledge thus gathered.

Figure 1

Some Compounds Isolated From P. brevi-compactum



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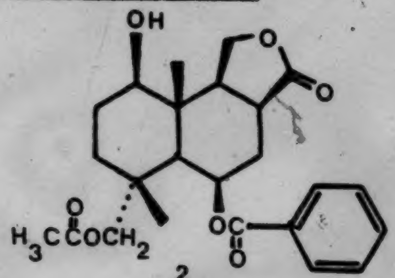

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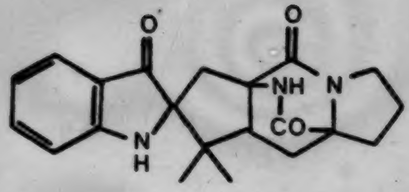

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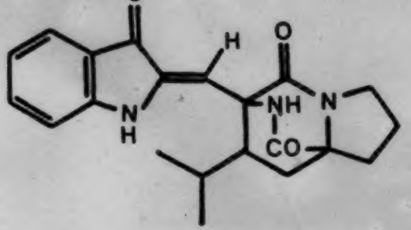

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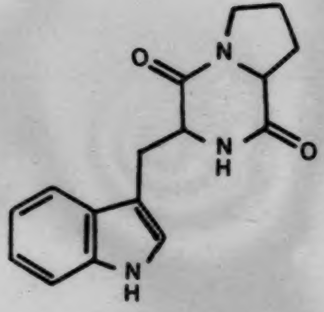

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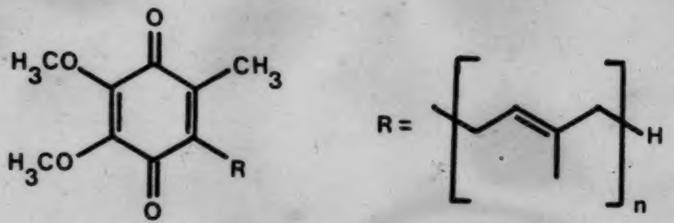
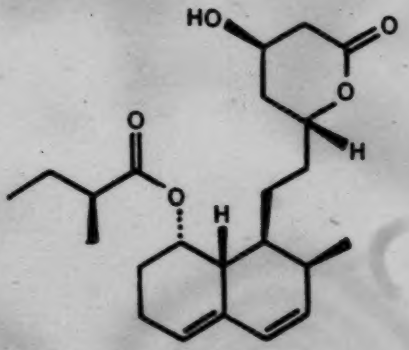


6

n = 7,8,9



7

DISCUSSION AND RESULTS

Part I Penicillium brevi-compactum (Dierckx)

Initially a 500 mL shake culture of P. brevi-compactum was used to inoculate still cultures grown in 1L Fernbach flasks. Of several media investigated the fungus grew best on a defined medium, Czapek SM, which gave the highest yield of crude organic extract. It grows quickly, covering the surface of the medium in less than a week.

A time study was performed to see if there was an optimum period of growth that would give the best yield of organic extract and/or highest biological activity. The maximum yield occurred at day 25 after innoculation but biological activity was best in a seven day period between days 16 and 23 after inoculation. The bioactivity was measured by observing the extracts' antifungal activity against the fungus Candida albicans.

Generally, the mycelium was separated from the medium and then extracted with ethyl acetate (EtOAc). Ca. 0.5 g of organic extract was obtained from 2 L of medium and ca. 0.2 g from the associated mycelium. Thin layer chromatography (tlc) of the medium and mycelial extracts, eluting with different methanol (MeOH), acetic acid (AcOH) and chloroform mixtures showed that these extracts differed significantly only in that the mycelial extract contained more non-polar materials in addition to a lot of fatty acid type material.

We also grew P. brevi-compactum in a 10 L fermentor. Comparison of the natural products identified from both modes of growth showed that there was only one that was unique to the still culture. This one will be discussed first.

1. 1-Deoxypebrolide (8)

Compound 8 was isolated in minor quantities (*ca*. 10 mg from 10 x 1 L still cultures) but was easily detected on tlc since it was visible under uv light and caused a pink/purple colouration when sprayed with vanillin/H_2SO_4 followed by heating.

The high resolution mass spectrum (hrms)* showed a base peak at 105 and other significant peaks at m/e 249, 292 and 309. These were consistent with fragments having the following formulae; C_7H_5O, $C_{15}H_{21}O_3$, $C_{17}H_{24}O_4$ and $C_{17}H_{25}O_5$, respectively. The mass losses from 309 to 249 and 292 to 232 indicated the presence of an acetate[22, 23] moiety which is supported in the proton magnetic resonance (^1H nmr) spectrum by a methyl singlet at 2.06 ppm[24] and a strong carbonyl absorption in the infrared (ir) spectrum at 1738 cm^{-1} and a C-O stretching absorption band at 1249 cm^{-1}[25, 26]. Similarly, a benzoate moiety can be assigned to the base peak (C_7H_5O) in the mass spectrum, and the aromatic protons at 7.96 ppm (the *meta* hydrogens) and 7.46 ppm (the *ortho* and *para* hydrogens) in the ^1H nmr spectrum and to a carbonyl absorption band at 1713 cm^{-1} and C-O stretching vibrations at 1278 and 1109 cm^{-1} in the ir, all typical for benzoates. The ultra violet (uv) and carbon-13 nuclear magnetic (^{13}C nmr) spectra are also in agreement.

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*The composition of all parent and fragment ions reported were determined by hrms. Because of the small quantities involved, elemental analyses were not performed except where specifically stated.

This information combined with other observations in the ir and ^1H nmr spectra led us to believe that the hrms had not indicated the parent mass so chemical ionisation mass spectroscopy (cims) was undertaken. This revealed the main peak at mass 432 which was consistent with a parent mass of 414*. Thus, if one considers the peaks in the hrms at 105 and 309 to be a result of facile loss of the benzoyl from the parent, the molecular weight of Compound 8 is 414 with the formula $C_{24}H_{30}O_6$.

This formula indicates a level of unsaturation equal to ten double bond equivalents. Six of these are accounted for by the acetate and benzoate moieties. A further two are satisfied by the presence of a γ-lactone. This is indicated most directly by the ir spectrum, carbonyl stretching vibration at 1779 cm^{-1}[26], and by a carbonyl carbon at 177.2 ppm in the ^{13}C nmr spectrum[29]. This leaves only two double bond equivalents which must mean two further rings since the ^{13}C nmr spectrum does not show signals, other than those for benzoate, where unsaturated carbons usually appear[30].

In summary, compound 8 consists of acetate and benzoate ester moieties and a 15 carbon segment which has two rings and a γ-lactone. All the oxygen atoms and unsaturations have been identified leaving only

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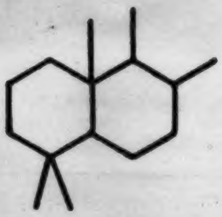
*This procedure involves introducing ammonia with the sample usually giving rise to a parent + NH_4^+ mass peak[27, 28].

the nature and substitution pattern of the 15 carbon segment to be assigned.

At any time that there appears to be a 15 carbon unit in a molecule the presence of a sesquiterpenoid skeleton can be suspected, especially when coupled with the observation of five possible methyl group equivalents. In our case ^{1}H nmr shows three methyl singlets at 2.06, 1.42 and 0.98 ppm (one being the acetate methyl), two methylenes attached to oxygen at 4.26 and 3.87 ppm, and the carbonyl carbon of the γ-lactone. It turns out, that there are only three common sesquiterpene skeletons which fit the requirement[31] of having five methyl groups and two rings: drimane (9), the tutins (10) and the skeleton of the fukinanolides (11), if we omit skeletons that have ethyl groups.



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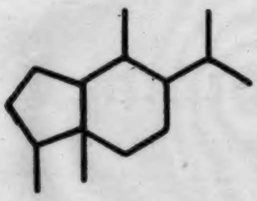

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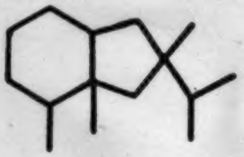

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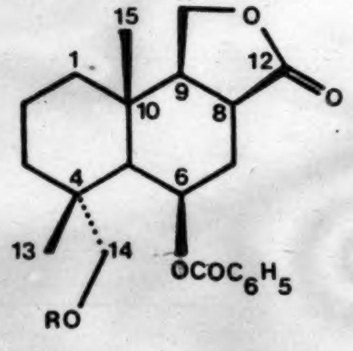
Further partial structures were obtained from the ^1H nmr spectra and by spin decoupling and lanthanide shift experiments on compound 8, its deacetylated derivative (8a), and the deacetyl debenzoyl derivative (12). The deacetyl derivative was obtained by treating compound 8 with aqueous methanol containing triethylamine. In the ir a broad band appeared at 3480 cm^{-1} while those at 1738 and 1249 cm^{-1} disappeared. The acetate methyl in the ^1H nmr also disappeared while one of the oxygen bearing methylenes shifted upfield by 0.45 ppm, as expected. The diol derivative (12) was obtained by more vigourous hydrolysis in 2N NaOH solution. Both hrms and cims showed peaks consistent with the hydrolysis of both acetate and benzoate units viz. masses for a parent + H at 269 (268 + 1) ($C_{15}H_{25}O_4$) in the high resolution mass spectrum and 286 ($M + NH_4^+$) and 554 ($2M + NH_4^+$) in cims. The ir shows the disappearance of the significant benzoate absorptions at 1713, 1278 and 1109 cm^{-1} as well as those for the acetate. The ^1H nmr spectrum agrees with this and also shows that there are two isomers present in the ratio 9:5 (12a and 12b respectively).

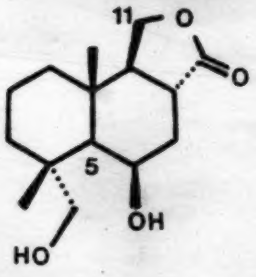


8 R = $COCH_3$
8a R = H

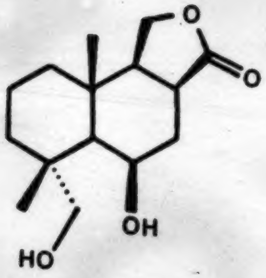
The information available from the initial 100 MHz spectrum of the natural compound (8) and the hydrolysis experiments may be summarized as follows:

The acetate is attached to a methylene (H_2-14 at 3.87 ppm) which appears as a simple AB quartet (^2J = 12Hz) indicating that it is further attached to a quarternary carbon. The benzoate is attached to a methine (5.70 ppm, H-6) which appears as a broad signal (W1/2 = 8Hz) indicating that it is adjacent to hydrogen bearing carbons. The final oxygen bearing carbon in the ^1H nmr (H_2-11 at 4.26 ppm) shows an AB quartet (^2J = 9Hz) with one of the doublets split once again into doublets (^3J = 5Hz) indicating that it is adjacent to a methine carbon.

More detailed coupling data was obtained on the diol derivative, compound 12a, the spectrum of which could be separated from that of its isomer, compound 12b, by spin decoupling experiments on a 400 MHz nmr instrument. This allowed us to link carbon-5 sequentially to carbon-11

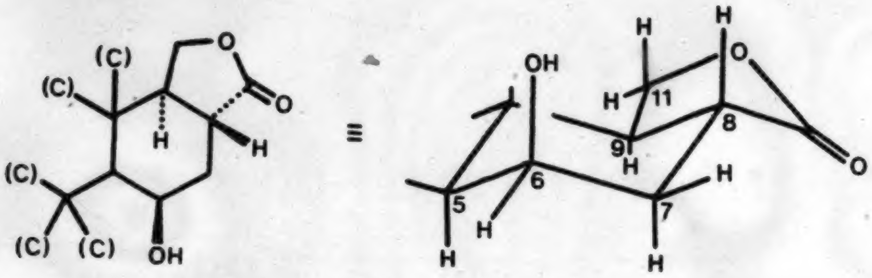


12a



12b

and also gave the relative stereochemistry. This is best explained in terms of the derived partial structure 13.



13

H-5 is coupled to H-6 by 2Hz which is in turn coupled to H-7ax and H-7eq by 3Hz. The small magnitude of these coupling constants indicates that H-6 is equatorial, assuming that these protons are in a constrained system, this being the only way no trans diaxial hydrogen couplings (^3J = 6 - 14Hz) could come about. H-7ax, (2.17 ppm) and H-7eq (1.59 ppm) are coupled geminally with ^2J = 12Hz. H-5 appears as a doublet at 1.27 ppm and is therefore attached to two quaternary carbons.

H-8 is also coupled to H_2-7 with coupling constants of 14 and 3.5 Hz clearly putting it in an axial orientation which is further supported by its coupling of 12.5 Hz to H-9, once again being a trans diaxial coupling. H-8's chemical shift at 2.81 ppm indicates that it must be α to the carbonyl. It is perhaps a little further downfield than normal but it is the only position that could produce a sufficient downfield shift which has not already been accounted for.

Lastly, the H-9 methine (1.93 ppm) is coupled only to the H_2-11 methylene in addition to the methine H-8 so the fourth substituent must be a quarternary carbon. Unlike the case of the natural compound, H-9 is coupled to both H-11α and H-11β by 11 and 7Hz.

This information, with the fact from the ^{13}C nmr spectrum that there are only two sp^3 quaternary carbons (36.6 and 34.2 ppm) gives rise to partial structure 13.

This partial structure is only satisfied by the drimane skeleton (9), one of the three skeletons postulated.

As the coupling constants in 13 and thus compound 12a are substantially different than in the natural compound (8), particularly the protons at carbons 8, 9 and 11, we feel that the proper orientation of the bonding in the natural compound (8) is as shown in structure 12b. Epimerisation at the carbon-8 under the basic hydroysis conditions satisfactorily explains our observations.

As we progressed in our study of compound 8 we became aware of a group of compounds reported by McCorkindale and coworkers[14], one of which, 1-deoxypebrolide (8), seemed to have a structure consistent with our observations. Professor McCorkindale kindly provided a copy of the ^{1}H nmr and ir spectra of 8 and we found that these coincided with our equivalent spectra. There is a slight discrepancy between McCorkindale's reported melting point and ours, 171 - 173° C[15] and 164 - 166° C, respectively. However, we do not feel that this is significant.

McCorkindale's final proof of the structure of pebrolide (2) rests on the x-ray structure determination of its 1-bromoacetate derivative[14] which was in turn correlated with the related 1-deoxypebrolide (8)[14, 15].

In the course of our studies, prior to our having access to the 400 mHz nuclear magnetic resonance spectrometer, we studied the relative change in chemical shift of the protons in deacetyl-1-deoxypebrolide (8a) under the influence of increasing concentrations of an europium shift reagent*. The europium shift reagent causes downfield shifts, the greatest occurring to those protons which are closest to the site(s) of complexation of the reagent. In this case we expected this site to be the primary alcohol at C-14.[32]

Figure 2 gives the results of this experiment in the form of a graph of change in chemical shift versus the amount of Eu(fod)$_3$ added. In this qualitative experiment the results confirm that the

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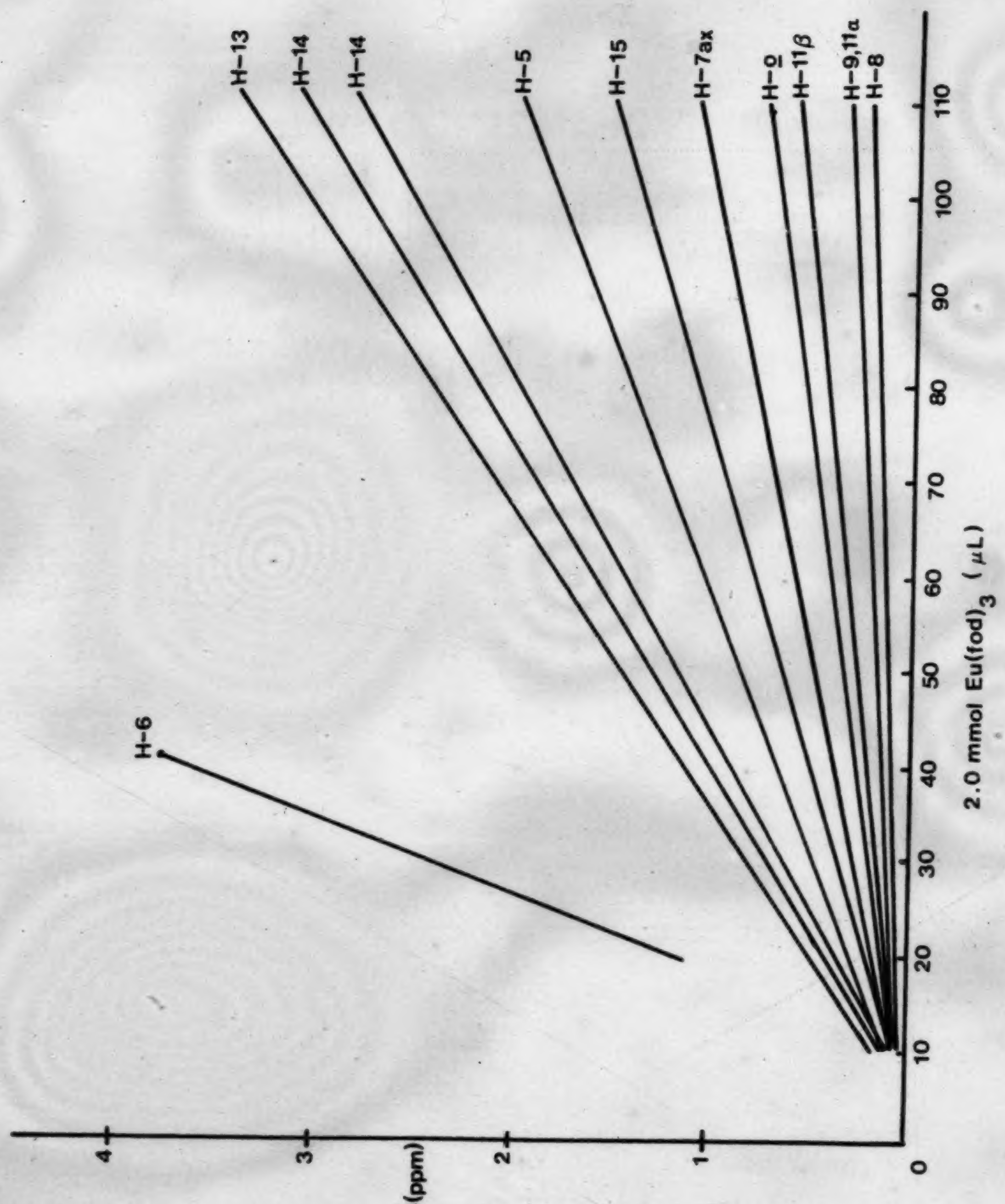
*tris-1,1,1,2,2,3,3-heptafluoro-7,7,dimethyl-4,6-octanedionato-europium (Eu(fod)$_3$).

Figure 2

Rate of Change of ^{1}H nmr Chemical Shifts of Compound 8a Upon Addition of $Eu(fod)_3$



acetate is on the α-methylene (C - 14) as the H-5 and H-6 methines are shifted more rapidly than the C-15 methyl, any of the benzoate hydrogens, or the H-2_{ax} hydrogen.

As mentioned previously, P. brevi-compactum was also grown in a fermentor. This procedure has several advantages in that the fungus grows a maximum amount of mycelium in a much shorter time viz eight days versus four weeks in a still culture. As this method changes the growing conditions considerably (the mixture is stirred and aerated), a different spectrum of metabolites may be expected. For this reason the extracts were again studied by tlc and bioassay. The following procedure was also developed in an effort to quickly locate any compounds that are biologically active.

The fermentor culture (ca. 10 L from one growth) was divided into 2.5 L portions and stirred repeatedly with EtOAc (ca. 500 mL) using a Hershberg stirrer. Approximately 10 grams of crude organic material was obtained from this extraction. The extract was divided into three equal portions, one portion was chromatographed on a silica gel column, the second was chromatographed on Sephadex LH-20 and the third was partitioned into strongly and weakly acidic, basic, and neutral compounds. The fractions thus collected were assessed by bioassay and the active ones were subjected to tlc. In this way we could select the compounds common to the active fractions and direct our isolation efforts accordingly.*

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*This procedure worked demonstrably well with the first fermentor growth but this turned out to be a special case the discussion of which, for coherency's sake, will be left till later.

Once this initial study had been completed the separation of organic compounds from the fungus was refined and, in fact, an alternative procedure to those already mentioned was used. The medium was filtered and the mycelium extracted using a Soxhlet extraction apparatus. The culture broth was concentrated in vacuo to ca. 1 L and extracted consecutively with toluene, ether, and n-butanol. Next, the extract was usually subjected to Sephadex LH-20 chromatography to remove the polypropylene glycol which had been added to the fermentation medium as an antifoaming agent*.

The toluene extract consisted largely of one compound (compound 14) which crystallised readily as colourless needles from benzene-petroleum ether (melting point of 143-145°C). The ease of separation of this compound was one reason why the liquid-liquid separation was considered an advantage.

2. Mycophenolic Acid (14)

The compound isolated from the toluene extract showed a parent peak at m/e 320 in hrms, having a molecular formula of $C_{17}H_{20}O_6$. The elemental analysis supported this formula (C 63.6%, H 6.26% found, C 63.74%, H 6.26 calculated) and indicates the presence of eight double bond equivalents in compound 14.

The ir spectrum (3426, 1730 and 1705 cm^{-1}) indicates the presence of alcohol(s) and two carbonyl functionalities.

When compound 14 was treated with diazomethane, two acidic

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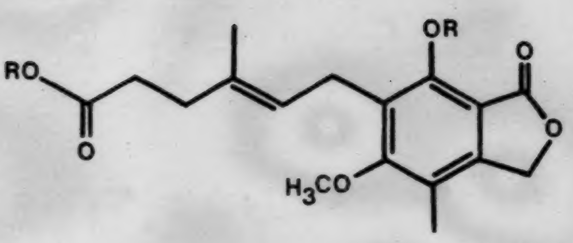
*Sephadex LH-20 chromatography is mainly a gel filtration process in which compounds are eluted according to their molecular weight and shape. The antifoam agent (MW ca. 2000) was eluted before any of the lower molecular weight metabolites of interest.



14 R = H

14a R = CH_3

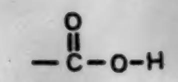
hydroxyls were methylated, forming compound 14a, as indicated by the ^1H nmr spectrum which shows two new methyl singlets at 4.03 and 3.59 ppm.

The ir spectrum of compound 14a shows no hydroxyl absorption band but shows carbonyl absorption bands at 1764 cm^{-1} (shifted from 1730 cm^{-1} in compound 14) and 1742 cm^{-1} (shifted from 1705 cm^{-1} in 14).

These data show the presence of a phenolic hydroxyl, a carboxylic acid and a lactone in compound 14. The assignment of the carbonyl functionality as a carboxylic acid (1705 cm^{-1}) and a lactone (1730 cm^{-1}) in compound 14 is supported by the ^{13}C nmr spectrum which shows two carbon signals characteristic of carboxylic acid or derivatives thereof at 175.1 and 172.9 ppm. The low absorption of the lactone carbonyl in the ir spectrum of compound 14 can be explained by hydrogen-bonding to the phenolic hydroxyl[33] since methylation to give compound 14a results in a shift in the ir spectrum of the lactone carbonyl to the higher, more usual, wave number.

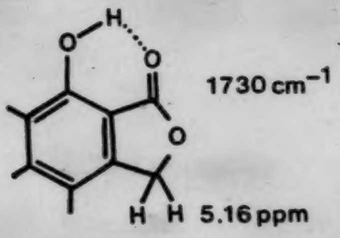
The ^1H nmr spectrum of compound 14 is remarkable in that it consists mostly of unconnected signals which then did not lend itself to extensive spin decoupling experiments. It shows no aromatic hydrogens. A three hydrogen singlet is present at 3.72 ppm which is consistent with a methyl ether as established inter alia by the methylation experiment. A two hydrogen singlet at 5.16 ppm can be assigned as being in the γ position of the lactone fused to an aromatic ring as shown in partial structure 15.

$Ar-CH_3$ 17

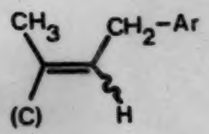


$-\overset{O}{\overset{\|}{C}}-O-H$ 18

$-O-CH_3$ 19



15



16

A further substituent on the aromatic ring was determined to be a methyl group which appears in the ^1H nmr spectrum as a three proton singlet at 2.12 ppm. Another methyl group appears at 1.78 ppm as a fine doublet (J = 0.5 Hz), the latter characteristic of a four bond, long range coupling. This chemical shift indicates that it is a vinylic group and thus attached to a fully substituted sp^2 carbon. This methyl group is coupled to a vinylic hydrogen (5.27 ppm, triplet of quartets (J = 7.0, 0.5 Hz) which is further coupled to a low-field methylene at 3.36 ppm.

The methylene chemical shift is considerably further downfield than normally observed for an allylic methylene (ca. 2.0 ppm) and thus must be adjacent to another group which causes a downfield shift. This fragment can be formulated as shown in partial structure 16.

Thus looking at the fragments identified--partial structures 15 to 19, all but two carbons are accounted for and all the oxygens and double bond equivalents have been designated. The ^{13}C nmr spectrum is completely in agreement with these assignments. We are able to assign the ^{13}C nmr signals to the fragments already formulated through their chemical shifts and multiplicities, as indicated by the off-resonance carbon-13 spectrum. This left us with two carbon triplets (i.e. two methylenes) corresponding to a four hydrogen multiplet observed in the ^{1}H nmr spectrum at 2.18 ppm.

These data suggested that compound 14 is mycophenolic acid, a relatively well-known natural product. An authentic sample of mycophenolic acid was obtained and a mixed melting point and the superimposition of the ir spectra confirmed the identity.*

The structure of mycophenolic acid remained obscure for some time after its initial isolation in 1932[7]. Raistrick and co-workers reported the basic structure in 1952 leaving in doubt only the configuration about the alkene double bond.[34] Total synthesis by Birch[35] and an x-ray crystal structure[36] was in agreement with the published structure and indicated that the double bond was trans.

This compound is regarded as a classical example of a natural compound which has a mixed biogenesis[37]. The aliphatic chain arises from the degradation of farnesyl pyrophosphate which is attached to the methylated aromatic portion of acetogenic origin followed by methylation of the phenol[38-41].

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*By identifying mycophenolic acid as a metabolite we were able to suggest the species name of the fungus which at that point had not been determined.

In our hands, mycophenolic acid showed remarkable antifungal activity against Candida albicans inhibiting growth of the fungus in a 1 cm radius zone around discs upon which mycophenolic acid had been adsorbed. However, when mycophenolic acid was tested against Ceratocystis ulmi it showed only minimal antagonistic action leading us to believe that it was not solely responsible for the activity.

3. N,N-dimethyl-3,6-dimethylthio-3-(4'-hydroxyphenyl methyl piperazine-2,5-dione (20)

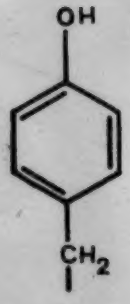
Compound 20 was isolated from the toluene extract along with mycophenolic acid. Compound 20 is also present in small quantities throughout numerous other growths of the fungus, both still and fermentor cultures.

By changing the ptlc solvent system from chloroform-methanol-acetic acid (89:10:1) for the final purification to toluene-acetone-acetic acid (69:30:1) better separation was obtained. Compound 20 is only faintly visible under uv light on the tlc plates but visualises yellow when sprayed with vanillin/sulphuric acid followed by heating.

Initially we had some problems in establishing the molecular weight of compound 20. The highest ion observed in hrms was at m/e 293 while the cims gave a number of masses including m/e 293 indicating that it was probably a fragment. It was initially difficult to obtain a reasonable mass match for some of the formulae proposed for the fragments, leading us to consider the presence of other heteroatoms in the molecule. The odour of the compound suggested that sulphur might be present and a small intensity peak (2.8%) at m/e 340 in one of several hrms spectra gave the molecular formula as $C_{15}H_{20}N_2O_3S_2$, which confirmed this. This matched with peaks at m/e 358 ($M+NH_4^+$) and 698 ($2M+NH_4^+$) in cims. The fragmentation pattern provided some significant information: an apparently facile loss of two methyl sulphide groups ($-SCH_3$), a distinct fragmentation pattern in which a fragment (wt 107) was lost leaving m/e 233, and a base peak at m/e 107. The 107 ion has a formula (C_7H_7O) which is usually associated with the presence of an hydroxylated benzyl fragment.

Indeed the ^1H nmr spectrum confirms the presence of an hydroxybenzyl fragment showing a distorted AB quartet at 6.91 and 6.70 ppm, typical of a benzene ring substituted at the para positions by alkyl and oxygen functionalities as shown in partial structure 21. Addition of D_2O did not change the ^1H nmr spectrum ($CDCl_3$). However when the spectrum was rerun in dimethylsulphoxide-d$_6$ (DMSO-d$_6$) a new one hydrogen signal appeared at 9.40 ppm. This indicates the presence of a phenolic proton.

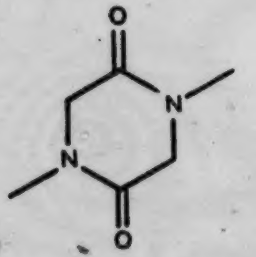
The ir spectrum shows a broad absorption band at 3350 cm^{-1} and a strong absorption band at 1665 cm^{-1} with a shoulder at 1657 cm^{-1}, consistent with the presence of phenol and possibly amide functions.



21

$2 \times CH_3-S-$

22



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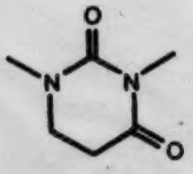
The ^1H nmr spectrum exhibits four methyl singlets, two at 2.16 and 2.26 ppm attributed to methyl groups on sulphur and supported by the loss of CH_3S fragments in the hrms of compound 20. The other two methyl signals in the ^1H nmr of compound 20 at 2.95 and 3.30 ppm suggested methyl amide substituents. It is of interest to note that the singlets at *ca.* 2.10 sometimes broadened out, at times sufficiently to become regarded as part of the background of the spectrum, disappearing altogether. The more downfield signal seemed to exhibit this behaviour to a greater extent. When the ^1H nmr spectrum of compound 20 was run in DMSO-d_6 the methyl sulphide signals did not appear broadened. This observation was not described by either Pettit[42] or Strunz[43] in their work with similar structure types, *e.g.*, gliovictin (25)[44].

This data suggests the partial structures 21 and 22 as well as methylated amide function(s) and probably no amine functionality. Removal of the hydroxybenzyl fragment from the molecular formula leaves $C_2HN_2(CO)_2(CH_3)_2(SCH_3)_2$ with one double bond equivalent unaccounted for. This means that "$C_2HN_2(CO)_2$" must be arranged in some cyclic format. Two common ways of arranging these atoms in a six membered ring as they occur in nature are the piperazine-2,5-dione (23) moiety and dihydrouracil (24). We prefer the piperazine-2,5-dione skeleton which



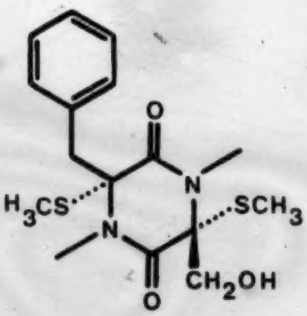
24

appears a significant number of times with sulphur substituents, e.g., gliotoxin (26)[45] and gliovictin (25)[44]. In addition, the carbonyl signals in the ^{13}C nmr of these types of compounds appear at 166 and 164 ppm similar to that of compound 27, a synthetic precursor of compound 20, whereas the carbonyl between two nitrogens has a chemical shift of 151 ppm[46].

The information presented suggests structure 20 for this metabolite. An isomer of compound 20, structure 28, can be eliminated as the structure of compound 20 since the ^1H nmr spectrum shows a clear AB quartet (3.48 and 3.08 ppm, ^2J = 14 Hz) for an isolated benzylic methylene group and a methine singlet at 4.17 ppm.

To verify the structure, compound 20 was treated with methyl iodide in refluxing acetone containing Na_2CO_3, in order to eliminate the methyl sulphide at carbon-3 and to methylate the phenol. Spectral evidence from the product indicated that the reaction proceeded as anticipated giving rise to compound 29.

The ir spectrum of compound 29 shows the loss of the hydroxyl



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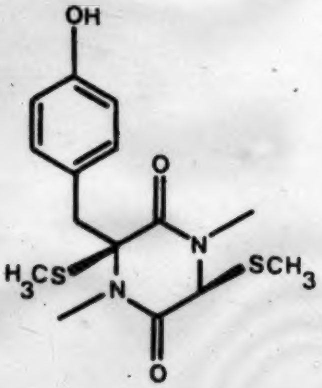

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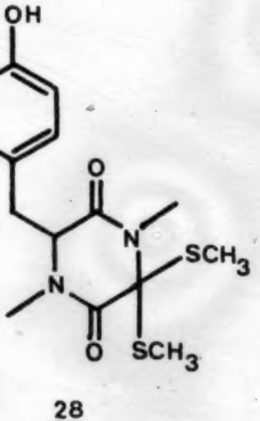

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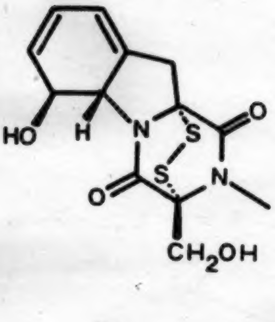

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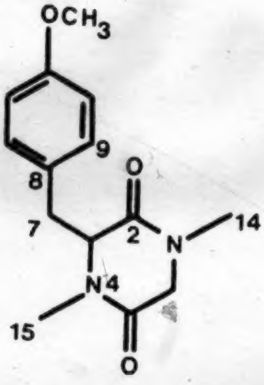

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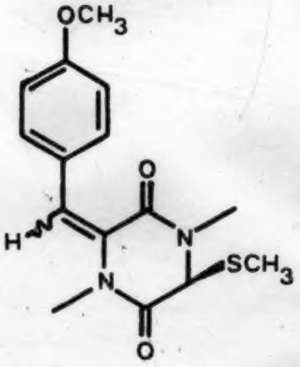

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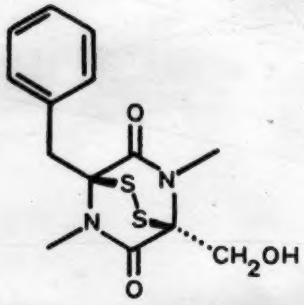


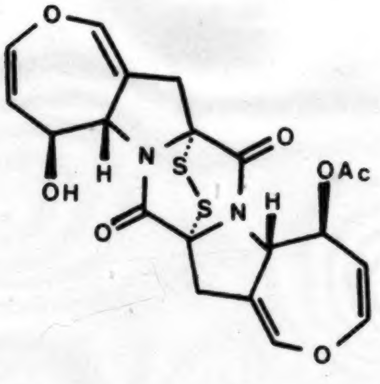
30

absorption band (at 3350 cm^{-1}). The formation of a methyl ether is indicated by a new methyl singlet at 3.83 ppm in the ^{1}H nmr spectrum. Elimination of methanethiol was evident from the hrms and the loss of the S-methyl signal (2.16 ppm) and the AB quartet, and the appearance of a one hydrogen singlet (7.16 ppm) in the ^{1}H nmr spectrum.

As a rule, members of this class of compounds, *e.g.*, 25 and 26, have the sulphur containing substituents in a *cis*-relationship, and thus it is reasonable to assume that the S-methyl groups are *cis* in the natural compound 20[48, 49]

The absolute configurations at carbons 3 and 6 or their equivalents have been found to be either both R or both S depending on the compound and sometimes on the source of the compound. Dorn and Arigoni isolated gliovictin from the fungus Helminthosporium victoriae[44] which had the 3R, 6R configuration and an $[\alpha]_D^{25} = -65°$ whereas Strunz et al. reported[43] the isolation of 3S, 6S gliovictin ($[\alpha]_D^{23} = +64°$) (as well as the epidithio parent, hyalodendrin (30)[47]) from an imperfect fungus of the Hyalodendron species.

The circular dichroic properties of natural epipolythiapiperazine-2,5-dione moiety-containing compounds[47] have been studied in an effort to find a way of predicting their absolute stereochemistry without x-ray analysis. Most of the analysis has been done on compounds which have the disulphide bridge and not the two methylthio groups. The disulphide bridge, which can be "right or left handed" depending on the configuration at the "3" and "6" positions has a major influence on the number and intensity of the peaks in circular dichroic measurements especially at higher wave lengths (i.e. $\geq$ 260 mμ). These have been used as the criteria to predict the stereochemistry of the bridged disulphide compounds. However, in cases where the bridge is opened and the sulphurs methylated these criteria are lost, leaving only the peptide $n \rightarrow \Pi^*$ transitions at ca 230 mμ of the piperazinedione system as significant and common peaks. There appears to have been some reticence in using the peaks at ca. 230 mμ as diagnostic in the epidithiapiperazine-2,5-dione compounds as the disulphide also has a generally stronger $n_2, n_3 \rightarrow \sigma^*$ charge transfer band in this region[50]. This aside, studies by Moncrief and Nagarajan in the aranotin (31) series establish some precedents upon which one can predict the stereochemistry of the dimethylpiperazinediones.



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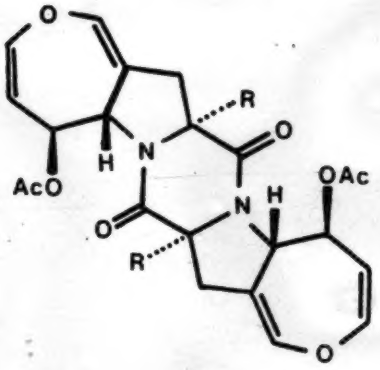

32 R = H
33 R = SCH$_3$

Acetylaranotin (31) which shows a highly negative circular dichroism curve (CD) at 229 mμ* was stereoselectively desulphurised with retention of configuration to compound 32 with Raney nickel[51].

Compound 32 shows a similar large negative Cotton effect at 222 mμ The CD curve of compound 32 was compared with the CD curves of two enantiomeric model compounds, L-prolyl-L-proline and D-prolyl-D-proline which give curves identical in magnitude but of opposite sign. De-epidithiaacetyl aranotin 32 gives a CD curve matching that of the L-prolyl-L-proline and thus is consistent with S configurations at the optical centres and furthermore indicates that aranotin itself has the R configurations at these centres. (Replacement of the hydrogens at the piperazine optical centres with sulphur substituents reverses the substituent priorities according to the Cahn-Ingold-Prelog sequence rule.) This conclusion is in agreement with the stereochemical assignment given for gliotoxin (26).

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*The whole CD curve was very similar to the one of gliotoxin (26) whose R, R configuration at sulphur is firmly established.

Nagarajan[52] has also indicated that bisdethiodi(methylthio) acetylaranotin (33) has the same negative Cotton effect at 230 mμ as aranotin indicating that its sulphur chiral centres should have the R configuration. Moncrief[53] confirmed this conclusion by an x-ray structure determination on the dimethylthio compound (33).

Thus there seems to be good correlations between the CD curves and the absolute stereochemistry within the compound types. Unfortunately complete information is not available on the compounds that most closely model ours viz the gliovictin enantiomers (25). CD information is available, however, on both enantiomers of hyalodendrin (30)[47, 53] through which their absolute stereochemistry was assigned. In addition, 3S, 6S-hyalodendrin (234 mμ, Δε +22.5) has been chemically correlated by reductive methylation to 3S, 6S-gliovictin isolated from the same fungus[43].

The other enantiomer of gliovictin, first isolated by Arigoni, could thus be shown to have the 3R, 6R configuration, as its specific rotation is opposite[44]. Unfortunately no CD information is reported for either enantiomer.

Another compound (34) co-isolated with compound 20 differs only by possessing an isoprenyl ether substituent at the phenolic position*.

We postulate that the absolute stereochemistry of compounds 20 and 34 is 3S, 6S based on the following information.

A model compound, (3S)N,N,O-trimethyl-3(4'-hydroxyphenyl) methylpiperazine-2,5-dione (27) which was synthesized from L-tyrosine

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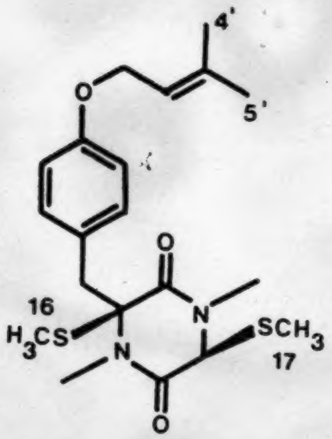
*The $[\alpha]_D$ for compound 20 is -28° versus compound 34 at -40° and their optical rotatory dispersion curves are similar.



34

ethylester shows a negative CD peak at 224 mμ(Δε -2.9) as expected for the n → π* transition of a piperazinedione[50]. The equivalent CD peak at 220 mμ of compound 34 is positive (Δε +2.8). On the basis of the foregoing discussion, the stereochemistry of both compounds 20 and 34 can be assigned as 3S, 6S.

4. N, N-dimethyl-3,6-dimethylthio-3-(4'-(3"-methyl-2"-buten-1"-oxy) phenyl) methylpiperazine-2,5-dione (34)

Compound 34 was co-isolated with compound 20 from the toluene extract of the fungal fermentation. It is less polar (tlc) and shows similar visualisation properties.

Determination of the molecular weight from hrms was again a problem and could only be inferred from the cims, the exact mass measurements of the fragments visible in hrms, and its obvious relationship to compound 20. The largest fragment in hrms appears at m/e 361 ($C_{19}H_{25}N_2O_3S$) which results from a parent ion of m/e 408 assuming the same facile loss of SCH_3 as observed for compound 20. This assumption is supported by the cims in which a mass of m/e 426 (i.e. 408 + NH_4^+) was observed and thus the molecular formula $C_{20}H_{28}N_2O_3S_2$ was assigned.

The major feature in the ir spectrum of compound 34 is again a strong absorption band for the amide carbonyls at 1665 cm^{-1}. The ^1H nmr spectrum of compound 34 is essentially identical with that of compound 20 having a para substituted benzene (7.00, 6.80 ppm 2H, AA'BB' J = 9Hz), two N-methyl singlets (3.26 and 2.98 ppm), a methylene AB quartet (3.58 and 3.09 ppm, ^2J = 14Hz), a methine singlet at 4.23 ppm, and two thiomethyl singlets at 2.16 and 2.28 ppm. Additional signals are observed: two broadened methyl singlets at 1.76 and 1.81 ppm, a methylene doublet at 4.48 ppm (J = 7Hz), and a broad methine triplet at 5.50 ppm (J = 7Hz). These signals can be assigned to an isoprenyloxy unit; methylene and methine are coupled to each other (J = 7Hz), typical chemical shifts for the methylene (4.68 ppm[24]) and vinylic methyls (1.50 ppm) are observed, and the expected long range coupling between the methine and the methyls is present. Comparison of the chemical shifts in the carbon spectrum of compound 34 with a known compound containing a similar fragment are in agreement[46].

Since the ir spectrum of compound 34 clearly shows the lack of any hydroxyl stretching absorption band, its mass spectrum loses a C_5H_9 fragment, and all the compound 20 hydrogens attached to carbon visible in its ^1H nmr spectrum are also present in the ^1H nmr spectrum of compound 34, it is reasonable to assume that an isoprene unit has replaced the phenolic proton of compound 20 to give compound 34. Such an inter-relationship is a frequent occurrence among compounds from natural sources.

Table 1 comparesthe carbon chemical shifts between inter alia

Table 1

Carbon-13 Shifts of the Piperazine-2, 5-dione Compounds

Compound Number	20	34	35	40	27
Carbon					
2*	166	165	166	167	166
5	164	164	165	163	164
11	156	158	158	160	159
10, 12	131	131	132	131	131
8	125	126	127	126	126
9, 13	116	115	114	114	114
3	76	75	67	85	64
methoxy	--	--	--	55	55
6	65	65	44	51	51
7	42	42	42	45	36
14+	34	33	--	33	33
15	31	30	--	27	32
17	16	16	--	--	--
16	14	14	13	--	--
3'	--	138	137	--	--
2'	--	120	120	--	--
1'	--	65	64	--	--
4'	--	26	25	--	--
5'	--	18	18	--	--

*2 and 5 may be interchanged.

+14 and 15 may be interchanged.

compounds 20 and 34. These show good agreement.

5. 3-Methylthio-3-(4'-(3"-methyl-2"-buten-1"-oxy) phenyl) methylpiperazine-2,5-dione (35)

Compound 35 was isolated from the very polar fraction of the toluene extract. Compound 35 is a white crystalline solid (melting point 205-207°C) and is quite polar having an R_f of 0.75 (1:10:89; Acetic acid-methanol-chloroform).

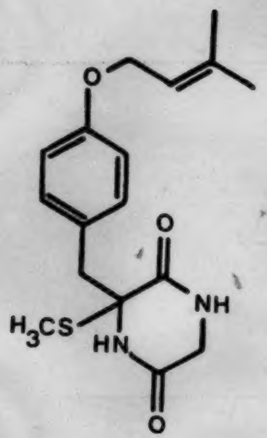
The ^1H nmr spectrum of compound 35 bears a superficial resemblance to that of compound 34 in that it shows the isoprenoxy moiety, thus leading us to suspect that this was a related compound.

This relationship is also evident in the hrms of compound 35 which shows the parent ion at m/e 334 (1%) (molecular formula $C_{17}H_{22}N_2O_3S$), lacking three methyl group equivalents and a sulphur atom with respect to compound 34. The fragmentation pattern agrees with the presence of isoprenyl and methylthio moieties.

The structure of compound 35 was readily assigned based on its ^1H nmr spectrum. No N-methyl groups are present and the downfield methylthio and the carbon-6 methine observed in the ^1H nmr spectrum of compound 34 are absent. These are replaced in the ^1H nmr spectrum of compound 35 by a methylene AB quartet one half of which is split into doublets (3.79 ppm, ^2J = 18Hz; 3.30 ppm, J = 3, 18Hz); a one hydrogen singlet at 8.84 ppm, and a broad one hydrogen singlet at 8.01 ppm.

The expected N-H stretching vibration absorption bands are present in the ir spectrum at 3180 and 3070 cm^{-1} and the amide carbonyl absorption band at 1688 cm^{-1} has shifted to shorter wavelength w.r.t.

compound 34.



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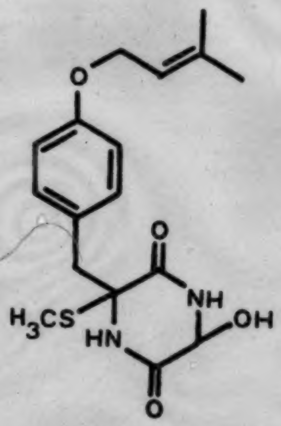
The carbon-13 spectrum whose chemical shifts are given in Table 1 are in accord with this structure assignment.

6. 6-Hydroxy-3-methylthio-3-(4'-(3"-methyl-2"-buten-1"-oxy phenyl) methylpiperazine-2,5-dione (36)

Compound 36 was isolated from the ethyl acetate extract of still cultures during the early part of our work.



36

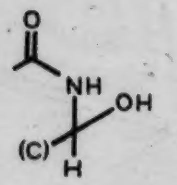
Initial attempts to propose a structure for this compound were unsuccessful since we were unable to determine a reasonable molecular formula for the compound. Our efforts were further hampered by the fact that we did not consider sulphur as a possible constituent of the molecule. Thus the problem was set aside for some time. Later data established the presence of piperazinedione metabolites in the extract from this fungus, but upon returning to this problem it was found that there was insufficient sample remaining to perform additional measurements except to repeat the hrms. With this we were able to assign its gross structure as shown in 36.

The hrms indicates a parent ion at m/e 350 which corresponds to the molecular formula $C_{17}H_{22}N_2O_4S$, one oxygen more than compound 35. The cims has peaks at 368 ($M + NH_4^+$) and 386 ($M + N_2H_8^+$). The same losses of CH_3S and isoprenyl are evident.

The film cast ir spectrum of compound 36 was remarkable in that it was dominated by an intense absorption band at 1679 cm^{-1} for the amide carbonyl stretching vibration. The hydrogen stretching vibration region in the ir spectrum shows six clear absorption bands at 3325, 3200, 3090, 3055, 2927 and 2880 cm^{-1} for the O-H, N-H and various C-H vibrations.

The ^1H nmr spectrum was taken in DMSO-d_6 due to the compound's low solubility and provided the bulk of the structural detail. Coupling between hydrogens was confirmed by spin decoupling experiments. A two hydrogen doublet at 4.44 ppm is coupled to a broad triplet methine signal at 5.39 ppm (J = 8Hz). This in turn exhibits long range coupling to two broadened methyl singlets at 1.73 and 1.68 ppm which suggests an isoprenoxy fragment. Coincident with the methylene at 4.44 ppm there is another signal *viz* a methine as a doublet of doublets

(J = 4 and 6Hz) coupled to an amide hydrogen at 8.61 ppm and an alcohol at 6.79 ppm. This spectroscopic data is consistent with a partial structure as shown in fragment 37. The remainder of the ^{1}H nmr spectrum displays a one hydrogen singlet at 8.82 ppm due to an isolated 2° amide (38), a four hydrogen AB quartet at 7.14 and 6.76 ppm due to a *para* substituted benzene ring and an isolated methylene (AB quartet at 3.08 ppm (14Hz), 39).



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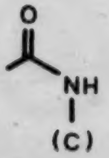

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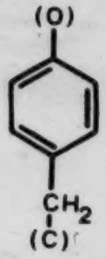


39

With this information, we postulate the structure of compound 36 as shown. Further work was precluded by a lack of material.

7. N,N-dimethyl-3-hydroxy-3 (4'-methoxyphenyl) methylpiperazine-2, 5-dione (40)

One other piperazinedione compound was isolated from the fermentation's toluene extract. Compound 40 (*ca.* 2 mg) was slightly less polar than compound 34 and shows familiar spectral properties.

The hrms shows a parent ion at m/e 278 for $C_{14}H_{18}N_2O_4$ (1%). (No signals for the methyl group of a thiomethyl function are present in the ^{1}H nmr and ^{13}C nmr spectra of compound 40.) Two useful fragmentations are observed: the base peak fragment (normally at m/e 107

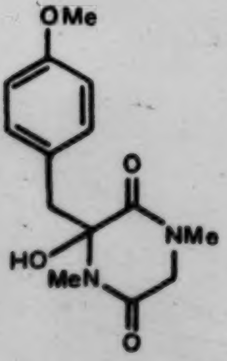
(C_7H_7O)) is at m/e 121 (C_8H_9O, i.e., replacement of a hydrogen with a methyl group), and the complementary fragment (m/e 157 (23%) $C_6H_9N_2O_3$), i.e., the N, N-dimethylated piperazinedione nucleus with an oxygen substituent.

The ir spectrum displays two significant absorption bands, one at 3360 cm^{-1} of an alcohol O-H stretching vibration and amide carbonyl absorption band at 1670 cm^{-1}.

The ^1H nmr spectrum shows the typical signals for a para substituted benzene, a benzylic methylene (AB quartet at 3.08 and 2.95 ppm, ^2J = 8Hz) and two N-methyls at 3.13 and 2.79 ppm. The methoxyl, a singlet at 3.78 ppm, is bonded to an aromatic ring. Support for this assignment is observed in the hrms and the ^{13}C nmr (55 ppm, the same chemical shift as observed for model compound 27). The alcohol substituent must be at carbon-3 as the benzyl methylene is not coupled further in the ^1H nmr spectrum and carbon-6 exhibits a clear AB quartet with signals at 3.39 and 2.34 ppm (^2J = 17.5Hz).

The synthetic compound (27) is almost identical to compound 40 lacking only the hydroxyl at carbon-3 and both ^1H nmr and ^{13}C nmr (see Table 1) spectra are consistent, taking the substituent effect of the hydroxyl into account.

This allows us to assign the structure of compound 40 as shown.



40

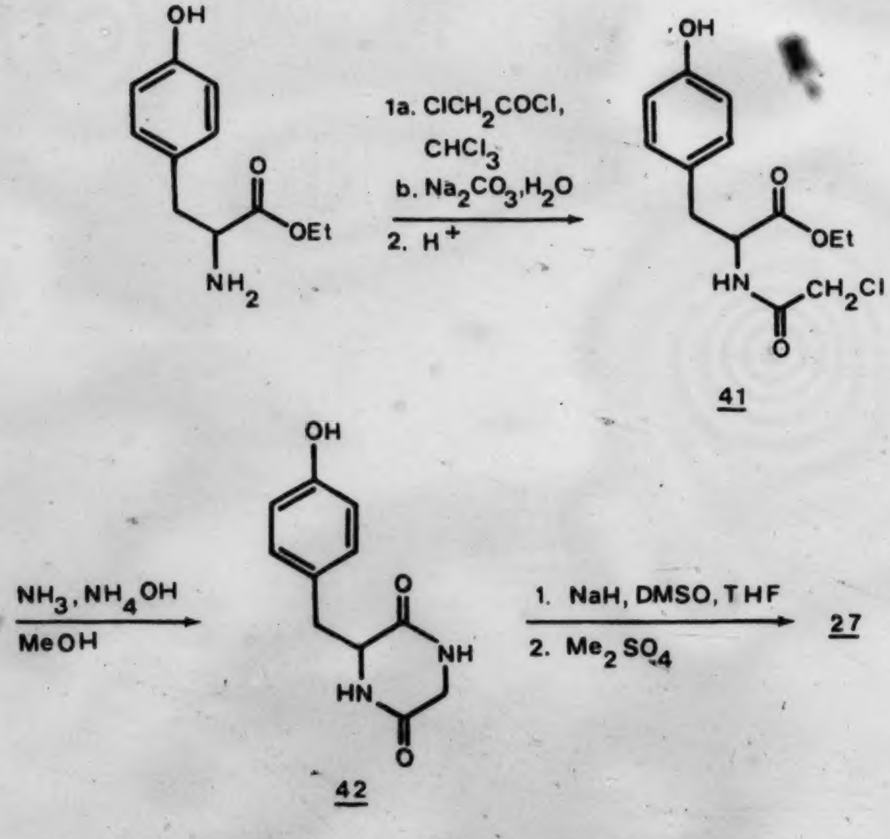
8. N, N-Dimethyl-3-(4^1-methoxyphenyl) methylpiperazine-2, 5-dione (27)

We found that compound 27 was useful as a model of the hydroxy benzylpiperazinedione nucleus for inter alia the assignment of the structures of several metabolites of P. brevi-compactum.

Compound 27 was synthesised by a method based on a procedure by Kawai[55] as indicated in figure 3. This procedure was selected as it by-passed problems of self condensation and protection/deprotection usually associated with peptide synthesis. L-tyrosine ethyl ester was reacted with chloroacetyl chloride to form compound 41. The structure of this intermediate was confirmed by the hrms and the ^{1}H nmr

Figure 3

Synthesis of compound 27



spectrum. An attempt to continue the synthesis by performing the ammonia condensation to complete the piperazinedione ring in "one pot", after the initial reaction was not successful and led to a low recovery of compound 41 (20% yield). Thus compound 41 was treated with ammonia in ammonium hydroxide/methanol and allowed to stand at room temperature in a pressure flask for three days forming compound 42 quantitatively. The hrms of compound 42 indicates the correct molecular formula (parent peak m/e 220, $C_{11}H_{12}N_2O_3$). The ir spectrum shows a carbonyl absorption band at 1665 cm^{-1}. The ^1H nmr spectrum (DMSO-$\underline{d}_6$) shows a phenol hydrogen at 9.24 ppm, an amide hydrogen (8.02 ppm, J = 3Hz) coupled to a methylene hydrogen (3.33 and 2.71 ppm; J = 17, 3 and 17 Hz, respectively), a benzyl methylene as an AB quartet (2.99 and 2.73, J = 14Hz) resplit into doublets (J = 4,5Hz, respectively). The carbon-3 methine (3.96 ppm) appears as a multiplet coupled to the benzylic methylene and the nitrogen-4 amide hydrogen at 7.79 ppm (2Hz). The para substituted benzene hydrogens occur at 6.94 and 6.64 ppm.

Compound 42 was treated with sodium hydride in tetrahydrofuran and DMSO, then methylated with dimethyl sulphate giving the trimethylated compound (27) in 74% yield. All the spectral data for compound 27 is in agreement with the assigned structure: hrms showed the parent ion at m/e 262, $C_{14}H_{18}N_2O_3$; and three new methyl singlets appear in the ^1H nmr spectrum at 3.79, 3.06 and 2.74 ppm for the methoxyl and two N-methyl groups respectively. The ^{13}C nmr chemical shifts of compound 27 are given in Table 1.

* * * * * * * * * * *

None of these compounds (20, 34, 36) when tested against C. ulmi or C. albicans showed any appreciable antifungal activity. This was not entirely unexpected as it has been widely reported[48, 49] that only those piperazinedione compounds which possess the disulphide bridge (e.g. 26, 30, 31) exhibit useful antifungal action.

A good example of this phenomenon is observed in the case of hyalodendrin (30) and gliovictin (25). Stillwell and co-workers reported the antibiotic activity of hyalodendrin (30) in 1974[56]. Hyalodendrin (30) was tested against 55 fungi, all of which were found to be sensitive at quite low concentrations, 50 μg per disc*. Significantly, from our point of view, hyalodendrin showed that it inhibited fungi associated with decay and deterioration in trees and wood products including C. ulmi and Gremmeniella abietina.

Stillwell found that 3S, 6S glioviction, i.e., the compound in which the disulphide bridge was replaced by thiomethyl ether groups and co-isolated with hyalodendrin, showed no antifungal activity. Thus the antifungal activity resides in the epidithiapiperazine-2, 5-dione moeity and modification at another site in the molecule, e.g. the acetate, did not affect its activity in bioassays.

Obviously in the search for the inhibitor of C. ulmi present in the strain of P. brevi-compactum which we possessed, there was the possibility that the active compound(s) do have the disulphide bridge. However, we did not manage to isolate any of the sulphur bridge compounds in our study. It is probable that the dimethylthio ether compounds are formed biogenetically from the disulphide bridge compounds since in an experiment feeding dimethylthio compound to a similar system none of it was incorporated in the epidithia compound[48]. In one attempt to screen for the presence of the disulphide bridge we employed tlc plates of the fungal extracts. The tlc plate was sprayed with 5% $AgNO_3$ solution which has been reported to show the disulphide compounds as black spots after a short period of time, whereas the thiomethyl compounds are visible only after twelve hours or so[48, 49]. This test showed only the methylthio ether compounds.

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*Their tests involved establishing a fungal colony on an agar plate and allowing the growing margin to advance toward 6 mm diameter paper discs impregnated with hyalodendrin.

Not withstanding these results, we still feel that it is reasonable to credit the presence of these epidithiapiperazine-2,5-dione with the bulk of P. brevi-compactum's antagonistic function toward C. ulmi. Our inability to isolate these compounds very likely is due to our method of growing the fungus. Strunz[54] considers the production of significant amounts of gliovictin from the hyaldendrin producing fungus to be caused by alteration in growth conditions, namely lengthening of the growth time. In our case it may be due to the more rapid growth induced by fermentation over that of still or shake cultures. If further investigation is carried out, presumably the fungus should be grown on agar plates upon which the initial antagonistic effect was most effectively observed.

9. (S)N-(1-hydroxymethyl-2-phenylethyl) benzamide (43)

Compound 43 was isolated from the still culture mycelial extract and purified by ptlc. This gave colourless crystals which when analysed by tlc could be visualised by uv light but did not char with vanillin/H_2SO_4 followed by heating.

The cims of compound 43 shows a major peak at m/e 256 ($M + H^+$) and a small one at m/e 273 ($M + NH_4^+$). The hrms shows the parent peak at m/e 255 which has the formula, $C_{16}H_{17}NO_2$. The base peak in hrms at m/e 105 (C_7H_5O) suggests the presence of a benzoyl group. Other fragments occur at m/e 164 (M^+ - C_7H_7) and 91 suggesting the presence of a benzyl group (91, C_7H_7). These two fragments (C_7H_5O, C_7H_7) account for most of the molecule leaving C_2H_5NO. Thus compound 43 contains no additional double bonds or rings.

The ir spectrum of compound 43 shows a broad absorption band at 3340 cm^{-1} and a carbonyl absorption band at 1637 cm^{-1} suggesting an amide and thus the benzoyl group must be part of a benzamide.

The ^1H nmr spectrum taken in DMSO-$\underline{d}_6$/$CDCl_3$ shows 10 aromatic hydrogens (7.82 ppm (2H), 7.40 ppm (3H), benzoyl; 7.24 ppm (5H), phenyl), a benzylic methylene (2.94 ppm as a doublet, J = 7Hz), an amide hydrogen (7.74 ppm as a broad doublet (J = 8Hz), and a primary alcoholic hydrogen (4.54 ppm as a triplet (J = 5.5 Hz) coupled to a methylene doublet of doublets (3.58 ppm, J = 4.5, 5.5 Hz). At 4.26 ppm there is a methine multiplet coupled to the benzylic methylene as well as the amide and primary alcohol (J = 8, 7, 4.5 Hz). This data allows us to deduce the structure of compound 43 as shown.

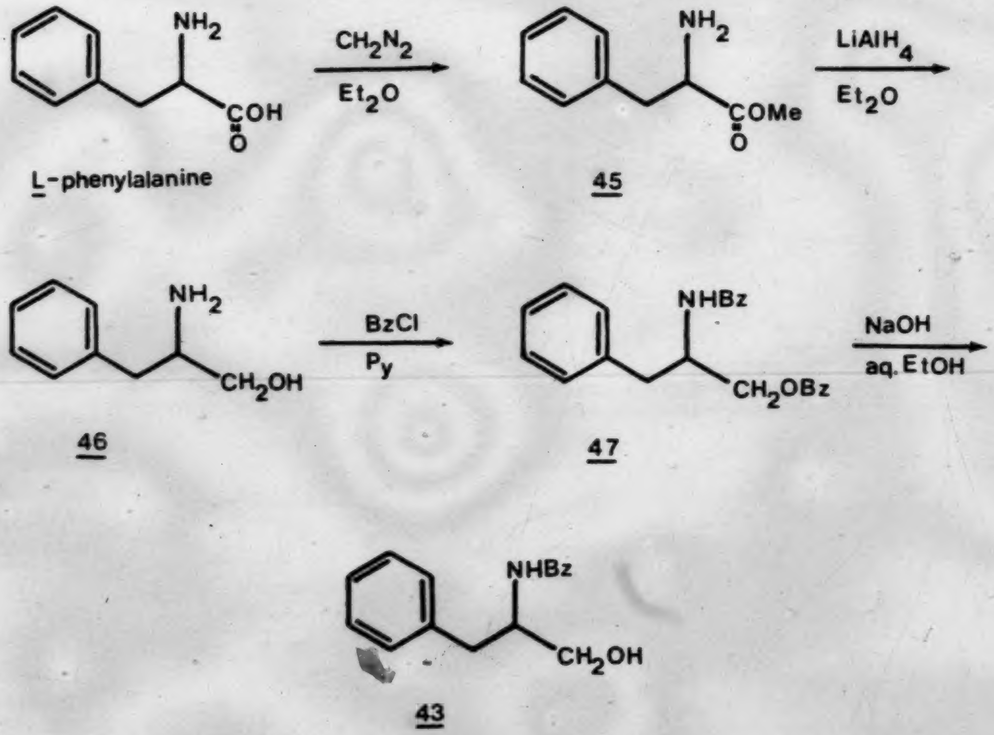
We found that N-(1-hydroxymethyl-2-phenylethyl) benzamide is a known compound, first reported in 1938 as a product of some synthetic studies[57]. (S)N-(1-hydroxymethyl-2-phenylethyl) benzamide, with a reported specific rotation of -78°[57], has since been isolated from two plant sources; Catharanthus pusilluss (Murr.)[58] and Alangium lamarckii[59] The specific rotation of compound 43 ($[\alpha]_D^{26}$ = -73°) isolated from P. brevi-compactum is in good agreement with that reported. As well, compound 43 has been found in the fungus Aspergillus flavipes[60] where it co-occurs with its oxidized dimer (44). Dimer 44 is a reported metabolite of Penicillium canadense[61].

It is interesting to note that when the primary alcohol of compound 43 is oxidised to an aldehyde, the aldehyde derivative is an effective inhibitor of α-chymotripsin[63]. Thus compound 43 may be indicative of another source of the bioactivity of *P. brevi-compactum*.

Comparison of the reported spectral data of *N*-(1-hydroxymethyl-2-phenylethyl) benzamide with that of compound 43 showed some ambiguities, thus we decided to synthesise compound 43 using established procedures[57, 58]. Figure 4 outlines the procedure used.

Figure 4

The Synthesis of (S)N-(1-hydroxymethyl-2-phenylethyl) benzamide



L-phenylalanine was esterified quantitatively by slow addition of CH_2N_2 in ether over a period of 60 hours at room temperature. The methyl ester 45 was reduced with lithium aluminium hydride (LAH) in ether. After one hour the reaction mixture was worked up and purified by chromatography. Recrystallisation from ether gave alcohol (46), mp 86-88°C (reported 85-86°C).

Both amine and alcohol functionalities of compound 46 were benzoylated by overnight treatment with benzoyl chloride in pyridine at room temperature. The crystalline dibenzoate (47) was selectively hydrolysed to compound 43 by treatment with sodium hydroxide in refluxing aqueous ethanol for one hour.

The product (43) was purified and decolourised. The spectral data and the melting point are consistent with the assigned structure. Synthetic compound 43 is enantiomerically pure having a specific rotation identical with that reported, $[\alpha]_D^{25} = -78°$[57].

Comparison of the ir spectra of the synthetic and naturally occurring compound 43 showed that they were superimposable, confirming the assigned structure of the natural metabolite.

10. Asperphenamate ((S)-N-benzoylphenylalanine-(5)-2-benzamido-3-phenylpropyl ester) (44)

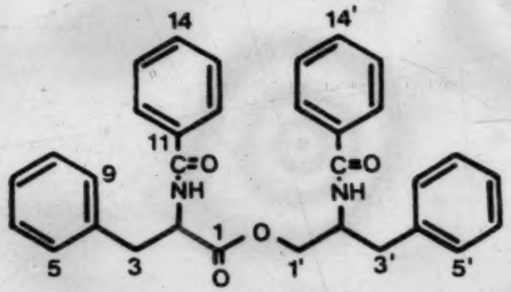
Compound 44, previously isolated together with compound 43 from A. flavipes[61] was isolated from the mycelial extracts of P. brevicompactum.

Compound 44, melting point 205-207°C (209-210°C lit.) $[\alpha]_D^{26} = -93.6°$ (c 1.01, pyr) is a comparatively non-polar compound (R_f 0.5 in AcOH:MeOH:$CHCl_3$, 0.5:5:94.5) which becomes almost intractable once it solidifies as a white solid. Purification of compound 44 was difficult due to co-crystallisation with a fatty acid.

Cims shows a small peak at m/e 507 ($M + H^+$). The hrms does not show the parent ion but shows the highest mass ion at m/e 415 ($C_{25}H_{23}N_2O_4$) arising from the loss of tropylium ion (m/e 91 (C_7H_7) 14%) from the parent, $C_{32}H_{30}N_2O_4$. Another important fragment at m/e 294 (6%) corresponds to the loss of benzamide from the 415 ion. Other ions in the mass spectrum appear at m/e 252 and 224 ($C_{16}H_{14}NO_2$, $C_{15}H_{14}NO$) corresponding to cleavage about the ester functionality, and at m/e 105, the phenylacylium ion, which is the base peak.

The ^{13}C nmr spectrum shows carbon signals in pairs indicating that compound 44 is a dimer of some sort. Table 2 compares the carbon chemical shifts of compound 44 with those of the monomer (43).

The ^1H nmr spectrum of compound 44 compares well with published data[60b]. The spectrum displays the following signals in $CDCl_3$/DMSO-d$_6$: 4 aromatic hydrogens as a multiplet at 7.78 ppm, 6 aromatic hydrogens at 7.35 ppm and two five hydrogen singlets at 7.19 and 7.23 ppm due to two phenyl groups. The carbon-2 methine hydrogen appears at 4.77 ppm as a broad quartet coupled to an amide hydrogen (8.52 ppm, d, J = 7Hz) and a benzylic methylene (3.18 ppm, d, J = 8Hz); the upfield signals due to the benzylic methylene are further split into a doublet (J = 3.5Hz). The carbon-2' methine appears as a broad multiplet at 4.46 ppm partially obscured by one half of the ester methylene (carbon-1') signal. The



44

Table 2

The Carbon-13 Chemical Shifts of Compounds 43 and 44

Assignment*	Chemical Shift (ppm)	
	43	44
1		171.7
1'	62.9	65.2
2, 2'	53.2	54.9, 50.3
3, 3'	36.5	37.0, 34.1
4, 4'	139.4	137.8, 137.1
5, 5', 9, 9'	127.2	127.5, 127.4
6, 6', 8, 8', 13, 13', 15, 15'	128.1	129.2, 128.4
7, 7'	125.9	126.7, 126.4
10, 10'	166.1	167.7, 167.2
11, 11'	134.8	134.7, 133.9
12, 12', 16, 16'	129.1	128.3, 128.1
14, 14'	131.0	131.5, 131.1

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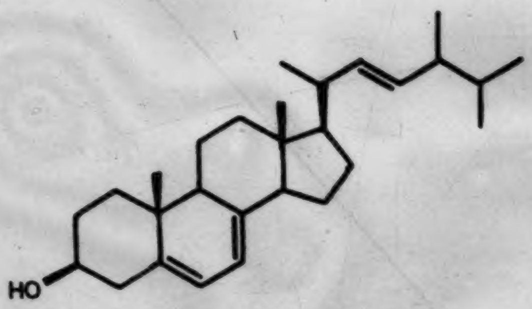
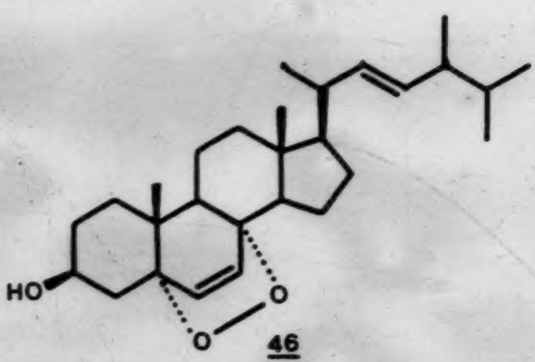
*The assignments in the range 128.0±1.6 ppm are tentative and may be interchanged.



45



46

ester methylene signal occurs as a pair of quartets centred at 4.40 and 4.02 ppm. Methine-2' is coupled to an amide hydrogen (7.96 ppm (J = 8Hz)) and a benzylic methylene (carbon-3', a doublet at 2.90 ppm, J = 7Hz).

The ir spectrum of compound 44 has five major absorption bands *viz.* 3300, 1638, 1530 cm^{-1} (2° amide), 1750 cm^{-1} (ester carbonyl) and 690 cm^{-1} (aromatic C-H bending vibrations) all as expected.

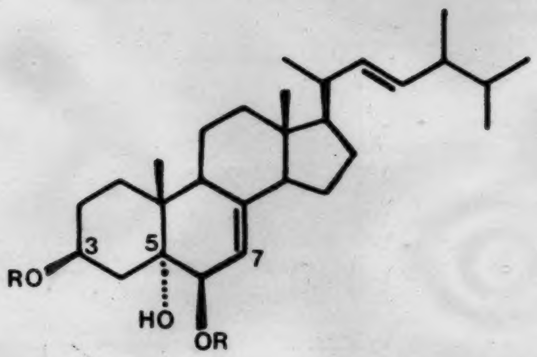
Comparison of the spectral data of compound 44 with published data[60, 61] established that compound 44 is identical with asperphenamate.

11. 3β,5α,6β,22E-Ergosta-7,22-diene-3,5,6-triol (cerevisterol) (47)

Ergosterol (45) and ergosterol peroxide (46) have frequently been isolated, in these laboratories and others, from the mycelial extracts of various fungi. Compounds 45 and 46 were also present in the mycelial extracts of *P. brevi-compactum*. Both compounds were identified by tlc and spectral data comparison with authentic samples.

One other steroid, tentatively identified as cerevistorol (47) has been isolated from the mycelium of both still cultures and fermentations of *P. brevi-compactum*.

The cims of compound 47 has a mass peak at m/e 448 corresponding to a parent ion 430 (*i.e.* M + NH_4^+). The parent ion ($C_{28}H_{46}O_3$) is absent in the hrms, the highest mass ion appearing at m/e 412 (26%) ($C_{28}H_{44}O_2$) as the result of the facile loss of a molecule of water. The dominant fragmentation pattern in the spectrum is the loss of up to three molecules of H_2O. The cleavage of the steroidal side chain (C_9H_{17}), typical of the ergosterol skeleton, can be inferred from peaks at m/e 269 and 251 whose formulae indicate the loss of a C_9H_{17} fragment and two or three molecules of water.



47 R = H
48 R = COMe

The ^{1}H nmr spectrum of compound 47 (DMSO-d_6) as well as spin decoupling experiments provided confirmatory information concerning the environment of the functionalised portions of the molecule. The two sp^2 hydrogens on the Δ^{22} double bond appear as a distorted triplet at 5.22 ppm. The tertiary alcohol -O$\underline{H}$ signal appears as a sharp singlet at 3.60 ppm. The carbon-7 sp^2 hydrogen, a broad doublet at 5.09 ppm (J = 6Hz) is coupled to the methine at carbon-6 (multiplet at 3.34 ppm) and to a secondary alcohol (doublet at 4.50 ppm (J = 5Hz)). The other secondary alcohol on carbon-3 appears as a doublet (4.23 ppm, J = 6Hz) and is coupled to a methine (broad singlet, W1/2 = 26Hz) at 3.78 ppm. The six methyl groups occur as two singlets at 0.55 and 0.91 ppm and four doublets at 0.80, 0.81, 0.88 and 0.99 (J = 6,6,7 and 7 Hz respectively). The remaining protons are visible as three broad unresolved signals centred at 1.90, 1.43, and 1.25 ppm.

The ir spectrum displays three absorption bands in the alcoholic O-H stretching vibration region at 3610, 3450 and 3310 cm^{-1} as well as a C=C stretching absorption band at 1659 cm^{-1}.

Acetylation of cerevisterol (47) with acetic anhydride in pyridine-chloroform gave the diacetate (48). The cims shows a peak at m/e 532 ($M + NH_4^+$) which corresponds to a molecular weight of 514. The higher mass fragments in the hrms are consistent with losses of acetic acid and water from the parent ion leading to m/e 376 ($C_{28}H_{40}$) as the base peak. The ir spectrum of compound 48 has a hydroxyl absorption band at 3450 cm^{-1} and two new carbonyl absorption bands at 1734 and 1713 cm^{-1}.

The ^1H nmr spectrum of compound 48 has signals for four hydrogens at 5.20 ppm, one hydrogen at 4.82 ppm as well as the two acetoxyl methyl singlets at 2.04 and 2.01 ppm.

Comparison of the data available on cerevisterol in the literature[63-66] with our isolate showed that they are in reasonable agreement[64] viz. melting point 257-259°C (256°C lit.), $[\alpha]_D^{26} = -75°$ (py) (-83° ($CHCl_3$) lit.); diacetate (48) melting point 165-166°C (171° lit.), $[\alpha]_D^{26} = -150°$ (py) (-146° ($CHCl_3$) lit.) as well as the ir and mass spectra[65, 66].

Cerevisterol (47) was first isolated and identified by Alt and Barton[63] as a minor sterol of yeast and has been isolated a number of times since from different sources. In at least two cases it was present with both ergosterol (45) and ergosterol peroxide (46)[65, 66].

* * * * * * * * * * *

As alluded to previously (page 18), we found that the metabolites isolated from our first fermentation culture appeared to be unique to that growth.

The ethyl acetate extract of the complete fermentation was separated into three portions. Each portion was further separated using Sephadex LH-20, silica gel chromatography or acid/base partitioning. Twenty-three fractions were obtained in this manner and these fractions were tested for bioactivity against C. albicans. Five of the fractions had very noticeable inhibitory effect on the test organism: two contiguous fractions (6 and 7) from the silica gel separation; the fourth fraction from the Sephadex LH-20 separation; and, the weak acid and neutral fractions. Three fractions contained the same metabolite (compound 62) when compared by tlc viz. the neutral, Sephadex and fraction 6 from the silica gel separation. The weak acid fraction contained p-hydroxybenzaldehyde (49) and the seventh fraction from the silica gel column provided two derivatives of anthranilic acid (50 and 57).

12. p-Hydroxybenzaldehyde (49)

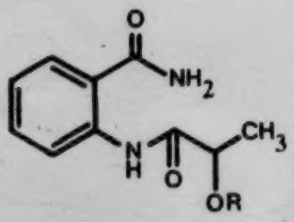
Compound 49 was isolated from the weak acid fraction of crude extract as described above followed by column and preparative thin layer silica gel chromatography.

It was identified as p-hydroxybenzaldehyde from its spectral data. Comparison of compound 49 with an authentic sample of p-hydroxybenzaldehyde (tlc, mp, superimposable ir spectra) confirmed their identity.

13. 2-(2'-Hydroxy-1'-oxopropyl) aminobenzamide (50)

Compound 50, which visualised as a bright blue fluorescent spot under uv light on a tlc plate, was isolated by ptlc (silica gel, $R_f = 0.15$ chloroform-methanol-acetic acid 89:10:1) from fraction 7 and purified by crystallisation from benzene-methanol (white crystals, mp 140-142°C).

This crystalline metabolite was assigned structure 50 based upon the following spectral and chemical data. The hrms shows the parent ion at m/e 208 (21%) with the molecular formula $C_{10}H_{12}N_2O_3$. The ^{13}C nmr spectrum of compound 50 has signals for six aromatic carbons (140, 133, 130, 125, 123 and 122 ppm) while the ^1H nmr spectrum shows signals for four aromatic hydrogens (carbons-3 and -6, 7.73 and 8.52 ppm, and carbons -4 and -5 at 7.14 and 7.49 ppm) consistent with an ortho-substituted benzene ring bearing at least one electron withdrawing group.



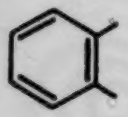
50 R = H
55 R = COMe

The ^{13}C nmr spectrum of compound 50 also shows two carbonyl signals (177 and 173 ppm) and the absorption band attributable to the carbonyl functionalities in the ir spectrum is at 1680 cm^{-1}, suggesting that the carbonyls are present in the form of amides.

When the ^1H nmr spectrum of compound 50 was determined in DMSO-$\underline{d}_6$ instead of methanol-$\underline{d}_4$, four one hydrogen signals became discernable. Each was a broad singlet. One of these was due to an alcohol since treatment of compound 50 with acetic anhydride in pyridine gave a mono-acetate derivative (55) (indicated by the loss of one labile hydrogen signal and the appearance of one acetyl methyl singlet at 2.25 ppm in the ^1H nmr spectrum of the derivative, as well as an ester carbonyl at 1740 cm^{-1} in its ir spectrum). Thus the remaining three labile hydrogens of compound 50 are likely due to a primary and a secondary amide.

Another fragment is readily discernible in the ^1H nmr spectrum of compound 50 as a methyl doublet (1.43 ppm, J = 7Hz) coupled to a methine quartet (4.24, J = 7Hz). The chemical shift of the methine is consistent with the presence of a secondary alcohol. The signal for the methine hydrogen in the ^1H nmr spectrum of the monoacetate 55 has moved downfield to 5.30 ppm, as expected.

These data suggest four fragments 51 to 54 which account for all the atoms in the molecule. Because the aromatic hydrogens at carbons -3 and 6 have significantly different chemical shifts (7.73 and 8.60 ppm) a structure which has both carbonyls adjacent to the benzene ring could be discounted. With this in mind, the most reasonable structure for compound 50 is as shown.



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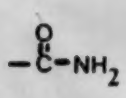
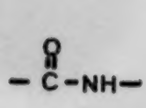


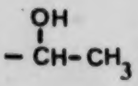
$-\overset{\overset{O}{\|}}{C}-NH_2$

52



$-\overset{\overset{O}{\|}}{C}-NH-$

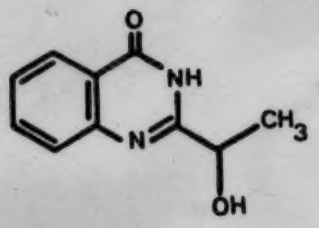
53



$-\overset{\overset{OH}{|}}{CH}-CH_3$

54

Further confirmatory evidence for the structure was obtained from the attempted hydrolysis of the monoacetate derivative 55. Compound 55 was stirred with 2N sodium hydroxide for ten minutes to give compound 56 after work-up. The hydrolysis reaction was monitored by tlc. The reaction seemed to take place in two steps, presumably first hydrolysis of the acetate followed by cyclisation.



56

The hrms of compound 56 shows the parent ion at m/e 190 (100%) ($C_{10}H_{10}N_2O_2$). This corresponds to the loss of a molecule of water from compound 50. The ^{1}H nmr spectrum of compound 56 shows that the hydroxyethanyl (54) and aromatic (51) fragments are still intact. Two of the labile hydrogens are no longer present. A reasonable explanation for the data is the formation of compound 56 since quinazolinones (or 4-hydroxyquinazolines) are readily formed by cyclisation of anthranilic acid derivatives.

14. 2-(1', 2'-Dioxopropyl) aminobenzamide (57)

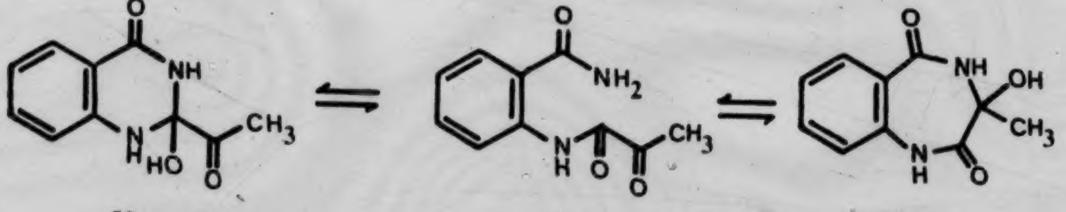
Compound 57 was isolated by ptlc (silica gel, R_f 0.4, chloroform-methanol-acetic acid, 89:10:1) as white, rectangular prismatic crystals, mp 198-200°C.

The spectral data obtained for compound 57 was quite similar to that of compound 50 and suggests that compound 57 is an oxidised form of compound 50.

The hrms of compound 57 shows a parent ion at m/e 206 ($C_{10}H_{10}N_2O_3$) while the ^{1}H nmr spectrum of compound 57 does not display a methine quartet due to a secondary alcohol. Its ir spectrum shows the amide carbonyl absorption bands at 1685 and 1655 cm^{-1} with an additional absorption band as a shoulder at 1710 cm^{-1}. This data allows us to assign compound 57 as shown, in which the secondary alcohol of compound 50 has been oxidised to a ketone.

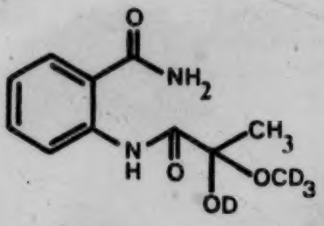
An interesting feature of the ^{1}H nmr spectrum of compound 57 (in MeOH-d_4) is that it appeared to have "shadow" signals which differ in intensity and chemical shift but have the same coupling patterns. A ^{1}H nmr experiment shows that this "shadow" phenomenon is temperature dependent, the upfield "shadow" signals being *ca.* six fifths the intensity of those for compound 57 at 10°C reducing to only one fifth at 60°C.

Initially this phenomenon was thought to be due to two slowly equilibrating tautomers of open or cyclic forms 58 $\rightleftharpoons$ 57 $\rightleftharpoons$ 59. However, this is not the case. Wegfahrt and Rapoport have previously studied



58 57 59

this system[67]. They found that when the ^1H nmr of compound 57 was determined in DMSO-d_6, a single, clean spectrum was obtained. The "shadow" signals observed when the ^1H nmr spectrum in MeOH-d_4 is best explained by the reversible addition of solvent to the dicarbonyl forming the hemiketal 60.



60

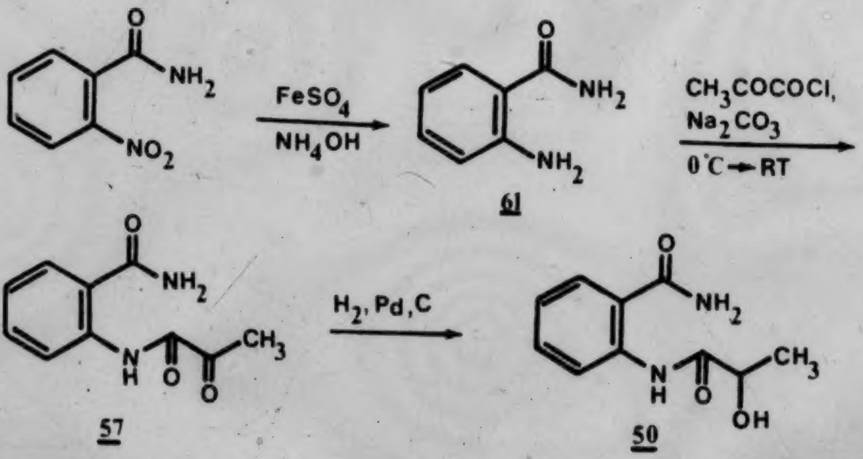
Compound 57 has previously been isolated by Suter and Turner[68] from the fungi *P. chrysogenum* and *P. notatum*, and by Kimura and co-workers from the culture filtrate of *Colletotrichum lagenarium*[69]. It has been shown to have activity as an antiauxin[70].

Since the amounts of compounds 50 and 57 isolated were small, we decided to confirm the identity by synthesis. Procedures utilizing anthranilic acid, causing it to react with pyruvyl chloride and then converting the N-pyruvyl anthranilic acid form to the primary amide (57) failed. The main problem was the formation of a 4-quinazoline compound.

This cyclisation was prevented by reversing the process, *i.e.*, making the primary amide first and then reacting it with pyruvyl chloride as shown in Figure 5. Thus *o*-nitrobenzamide was reduced to *o*-aminobenzamide (61) with $FeSO_4$[71], and this was then acylated with pyruvyl chloride (prepared according to the method of Ottenheijm and de Man[72]). The melting point, tlc, and spectral characteristics of the synthetic compound 57 were identical with the naturally occurring metabolite.

Figure 5

The Synthesis of Compounds 50 and 57



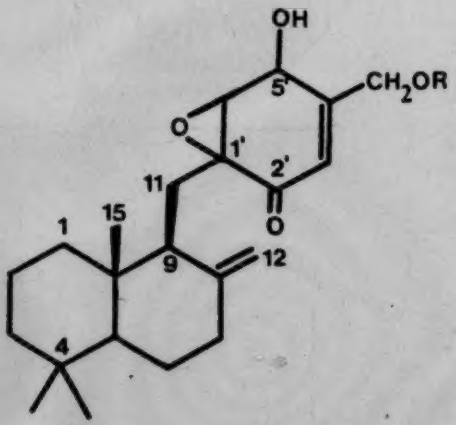
Suter and Turner[68] also reported the reduction of the ketone carbonyl of compound 57. When compound 57 was reduced with $NaBH_4$ the reduced and cyclised product (*i.e.* compound 56) was obtained, however catalytic hydrogenation of compound 57 gave the secondary alcohol 50. Treatment of synthetic compound 57 under the reported conditions (5% Pd/C;H_2 (1 atm); MeOH) gave a product which was identical (ir, hrms, ^{1}H nmr) with the natural compound 50.

15. The C_{22} Compound 62 (11-(1'-epoxy-5'-hydroxy-4'-hydroxymethylcyclohex-3'-en-2'-onyl)-$\Delta^{8(12)}$-drimene

As described (page 51), compound 62 was the major component of the bioactive fraction from the ethyl acetate extract of the fungus grown by fermentation. The bioactive fractions were recombined and chromatographed on a silica gel column to give compound 62 which contained a small amount of contaminant. The contaminant was removed by fractional crystallisation.

Compound 62 has an R_f of 0.45 on silica gel tlc (acetic acid-methanol-chloroform; 1:10:89) and visualises readily as a rich dark brown spot with H_2SO_4/vanillin. It is a white crystalline solid with a melting point of 124-26°C.

We have tentatively assigned the structure of compound 62 as shown based on analysis of spectral and chemical data and by the comparison of the spectral data of several known compounds containing similar structural moieties.



62 R=H

63 R=COCH$_3$

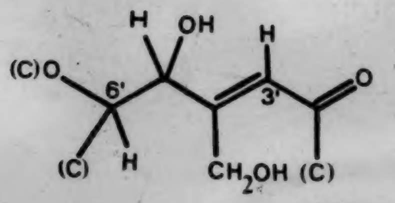
The mass spectra (hrms and cims) show that the molecular formula of compound 62 is $C_{22}H_{32}O_4$, and thus it possesses seven double bond equivalents. The ^{13}C nmr spectrum indicates the presence of three double bonds, viz an α, β-unsaturated carbonyl (194 ppm, singlet, C=O; 159 ppm, singlet; 120 ppm, doublet) and an exocyclic methylene (149 ppm, singlet; 107 ppm, triplet). Thus compound 62 must have four rings.

Additional evidence supporting the presence of an α, β-unsaturated ketone was obtained from the ir (1680 cm^{-1}) and uv (λmax 239 nm; within the calculated value for a β, β-disubstituted α, β-unsaturated enone[24]) spectra of compound 62 and through chemical correlations. The ir spectrum of the $LiAlH_4$ reduction product of compound 62 does not show bands in the carbonyl region. However catalytic hydrogenation of compound 62 gives a product the ir spectrum of which shows the presence of a ketone carbonyl (1710 cm^{-1}).

The ir spectrum of compound 62 also indicates the presence of hydroxyl groups (3400 cm^{-1}). Treatment of compound 62 with acetic acid in pyridine yielded a diacetate derivative (63) whose ir spectrum has no hydroxyl absorption bands. The ^{1}H nmr spectrum of compound 63 shows two acetyl methyl singlets at 2.19 and 2.08 ppm and significant downfield movement of a methylene and a methine signal (4.31 ppm down to 4.64 ppm and 4.57 ppm down to 5.84 ppm, respectively). This information suggests that compound 62 contains a primary and a secondary alcohol in its structure.

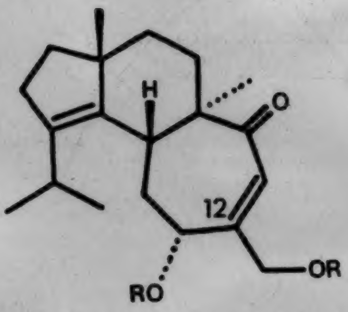
The ^{13}C nmr spectrum of compound 62 shows four carbons attached to oxygen, viz 65 ppm (doublet), 62 ppm (triplet), 61 ppm (doublet) and 61 ppm (singlet). Three of the four oxygens are already accounted for above, one carbonyl and two alcohols and therefore the other oxygen must be an ether attached to secondary and tertiary carbons.

Initially the ^{1}H nmr spectrum of compound 62 was difficult to interpret, however spin decoupling experiments of its diacetate derivative (63) aided in defining the partial structure shown as 64. Table 3 records the ^{1}H nmr chemical shifts used in the following discussion. In the ^{1}H nmr spectrum of compound 63 the 4'-methylene appears as a broadened AB quartet (4.77 and 4.56, ^2J = 15Hz) which is long range coupled to two one hydrogen signals at 6.04 and 5.84 ppm. The signal at 6.04 ppm is assigned as an α hydrogen on an α,β-unsaturated ketone by comparison to accepted data[24]. The one hydrogen signal at 5.84 ppm (carbon-5') is also coupled to a one hydrogen doublet at 3.75 ppm (J = 3Hz) and has been assigned to the 2° acetate methine. This was verified by comparison of the ^{1}H nmr spectra of the free alcohol 62, the diacetate 63 and the ketone 74. The ^{1}H nmr spectrum of the free alcohol shows the carbinyl hydrogen at 4.57 ppm. This signal moves downfield to 5.84 ppm in the ^{1}H nmr spectrum of 63 and is absent in the ^{1}H nmr spectrum of compound 74(p. 68). The signal at 3.75 in the ^{1}H nmr



64

spectrum of compound 63 can only be due to a methine with an oxy substituent adjacent to a fully substituted carbon. This information allowed us to draw a partial structure as proposed (64). Further support concerning the substitution environment at carbon-4' can be obtained from ^{13}C nmr spectra. In the diacetate (63) there is a large upfield shift of the signal assigned to carbon-4' from 159 ppm (singlet) in the ^{13}C nmr spectrum of compound 62 to 149 ppm in the ^{13}C nmr of compound 63. This is an unusually large shift for a carbon β to an alcohol but a similar large shift has been observed for the cyathin series of compounds, viz acetylation of cyathin A_3 (65) to the diacetate (66)



65 R = H
66 R = $COCH_3$

causes the sp^2 carbon (12) to move upfield from 153.9 ppm in the ^{13}C nmr spectrum of compound 65 to 143.4 ppm in the ^{13}C nmr spectrum of the diacetate (66)[73].

The exocyclic methylene hydrogens in the ^1H nmr spectra of compounds 62 and 63 possess discrete chemical shifts (4.57 and 4.81 ppm in 62 and 4.48 and 4.82 ppm in 63). These hydrogens are only coupled to each other (by a small coupling constant typical of geminal vinylic hydrogens) thus the adjacent sp^2 carbon must be fully substituted.

Table 3

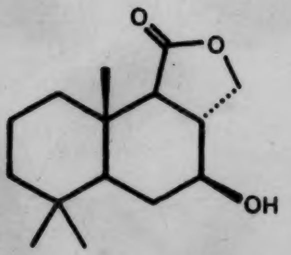
Assignable ^1H nmr Chemical Shifts of Compounds 62 and 63

Compound Solvent	62 $CDCl_3$	62 Py-d_5	62 DMSO-d_6	63 $CDCl_3$	74 $CDCl_3$	75 $CDCl_3$
H-3'	5.96	6.64	5.80	6.04	6.65	6.48
H-5'	4.57	5.06	4.53	5.84		4.98
H-12c*	4.57	4.85	4.53	4.48	4.53	4.55
H-12t	4.81	4.85	4.73	4.82	4.84	4.84
CH_2-4'	4.31	4.85	4.13	4.77, 4.56	4.56, 4.36	
H-6'	3.71	4.14	3.61	3.75	3.75	3.61
H_3-13✦	0.89	0.81	0.83	0.87	0.87	0.88
H_3-14	0.82	0.75	0.79	0.80	0.80	0.81
H_3-15	0.72	0.69	0.63	0.71	0.71	0.72
$C\underline{H_3}C$-O				2.19, 2.08		
$C\underline{H}O$						9.72

*H-12c is the vinylic hydrogen spatially closer to carbon-11.

✦H_3-13 and H_3-14 may be interchanged

Detailed inspection of the fragmentation pattern in the mass spectrum of compound 62 led us to conclude that the molecule was divided into two distinct parts: one with 14 or 15 carbons and a smaller fragment carrying all the oxygen functionalities. This provides further evidence for the partial structure 64. The fragmentation pattern of the 15 carbon portion is reminiscent of the fragmentation pattern of a drimane sesquiterpene, *e.g.*, compound 67[74]. Other drimane sesquiterpenes have been isolated from this fungus (*e.g.* 8). All of the major fragments

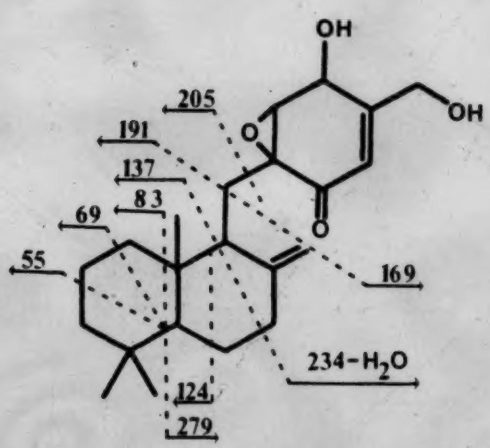


67

(>10%) containing only carbon and hydrogen and below m/e 138 in the hrms of compound 62 also appear in the mass spectrum of compound 67. There are 17 such fragments. The peak at m/e 137 ($C_{10}H_{17}$, 86%) is compatible with placement of the exocyclic double bond at carbon-8 since allylic cleavage can give rise to the fragment and is typical of such a system[75]. Figure 6 indicates some of the points of fragmentation of compound 62 observed in the hrms.

Figure 6

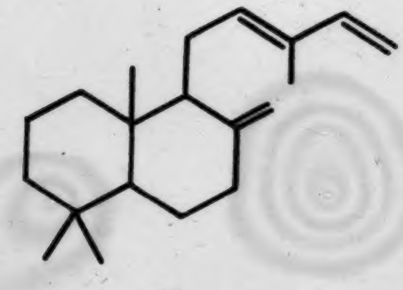
Hrms Fragmentation Modes of Compound 62



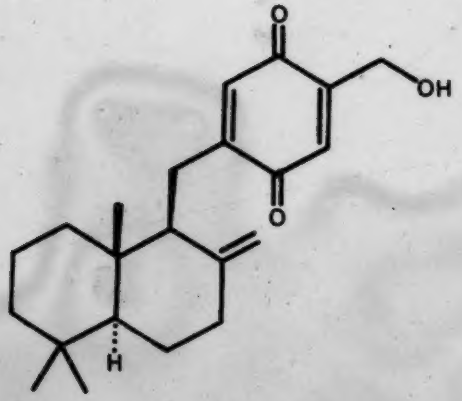
Compounds that have a drimene skeleton with a carbon-11 acetogenic or terpenoic substituent are relatively common. One such compound is the labdane derivative 68[76] reported by Bohlmann *et al*. The ^{13}C nmr spectrum of compound 68 shows good agreement with similar carbon signals in the ^{13}C nmr spectrum of compound 62. Table 4 compares their respective ^{13}C nmr chemical shifts. Significant deviations in chemical shift occur near carbon-9 as expected.

Combination of the two structural fragments drimene and 64 allowed us to assign the structure of compound 62 to be that shown. A very similar compound, tauranin (69)[77] has previously been reported.

Another known compound, epoxydon (70)[78], also known as phyllosinol[79], is very similar to the cyclohexene portion of compound 62. Figure 7 gives the structure of epoxydon and its assigned ^{1}H nmr chemical shifts and compares it with the ^{1}H nmr chemical shifts observed for the cyclohexene portion of compound 62. The dissimilarity between the chemical shifts of H-5 and H$_2$-7 of epoxydon and H-3' and CH$_2$-4' respectively is consistent with the inversion of the substitution



68

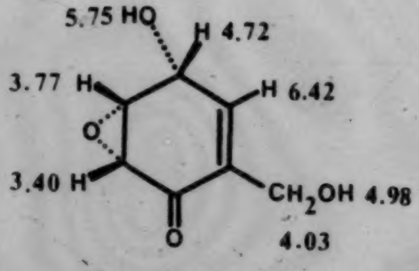


tauranin (69)

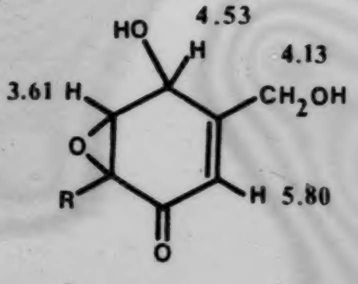
Figure 7

Comparison of the ^{1}H nmr Signals* and UV Spectra

of Epoxydon and Compound 62



epoxydon (70)



62

UV EtOH λmax(log ε) 239.5 (3.71)
nm 333.5 (1.65)

UV MeOH λmax(log ε) 221 (shl)
nm 239 (3.91)

Table 4

Comparison of the ^{13}C nmr Chemical Shifts of Compound 62 and Labdane Derivative 68

Compound Carbon (multiplicity)	62	68	Δδ
1 (t)	39.0	39.4	0.4
2 (t)	19.5	19.5	0.0
3 (t)	42.3	42.3	0.0
4 (s)	33.7	33.7	0.0
5 (d)	55.7	55.6	-0.1
6 (t)	24.7	24.3	-0.4
7 (t)	38.3	38.2	-0.1
8 (s)	149.4	148.6	-0.8
9 (d)	51.8	57.3	5.5
10 (s)	39.9	39.7	-0.2
11 (t)	21.3	23.3	2.0
12 (t)	106.8	107.5	0.7
13 (q)	21.8	21.8	0.0
14 (q)	33.6	21.8	0.1
15 (q)	14.7	14.5	-0.2

Chemical shifts are given in ppm downfield from TMS.

$CDCl_3$ as solvent.

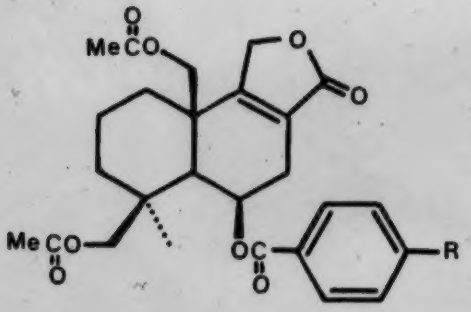
pattern about the double bond.

Some postulations can be made concerning the relative stereochemistry of compound 62. The extremely close correlation of the ^{13}C nmr spectra of compound 62 and the labdane derivative 68 especially in the A ring suggests strongly that the stereochemistry of the drimane portion of compound 62 is identical with that of the labdane compound. This relative orientation is the one most commonly observed in naturally occurring compounds containing this moiety. The stereochemistry which could be most easily altered is the carbon-9 to carbon-11 bond. We believe that this bond is β as is usual, since transformation of carbon-11 to an α orientation would remove one of the γ-gauche steric interactions on carbon-15 and would consequently be expected to produce a downfield shift of ca. 4 ppm of the carbon-15 signal in the ^{13}C nmr spectra. This downfield shift is not observed; carbon-15 differs by 0.2 ppm in the ^{1}C nmr spectra of compounds 62 and 68.

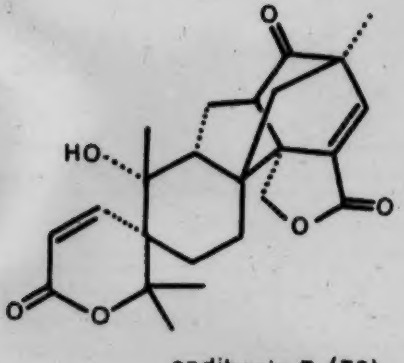
The stereochemistry of the cyclohexenone portion is difficult to ascertain from the information available. The absolute configuration of the epoxide can be suggested to be identical to that of epoxydon. The circular dichroism curves of both compounds 62 and 70 have positive Cotton curves at 334 nm and 341 nm respectively and negative Cotton curves at ca. 250 and 245 nm respectively[78].

Work on compound 62 was discontinued due to the lack of material and inability to isolate more from subsequent cultures of the fungus. Neither the remaining viable cultures nor a new culture obtained from the Northern Forest Research Centre appeared to produce compound 62 upon fermentation. There are two possible explanations for this. One is that the first culture contained a fungal contaminant which was more virile than P. brevi-compactum. Another is that compound 62 arose out of a mutant strain of P. brevi-compactum. We find the latter a more plausible explanation since we have already shown that the fungus has the biosynthetic capability to produce the drimane sesquiterpene skeleton.

Recently, Simpson reported[80] the structures of astellolides A and B (71 and 72 respectively) from a mutant strain of the fungus Aspergillus variecolor which, in its non-mutant form had provided andibenin B (73)[81]. Simpson speculated that in the mutation of A. variecolor the biosynthetic pathway that causes the formation of andibenin B through mixed biogenesis[82] had been cut off and the farnesyl pyrophosphate had been diverted to the production of the drimane skeleton of the astellolides. Simpson further observed that this appeared to also occur in the case of P. brevi-compactum which produces mycophenolic acid (14) by mixed biogenesis (a farnesyl and a acetogenic part which closely resembles the one in andibenin B) and also produces the pebrolide compounds, e.g., 2 and 8. The production of compound 62 seems to be



71 R=H
72 R=OH

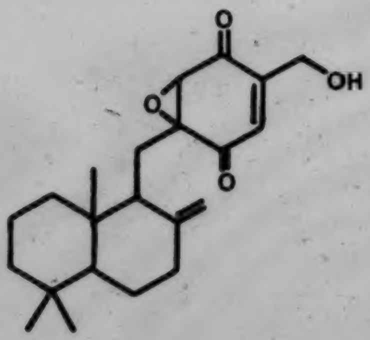


andibenin B (73)

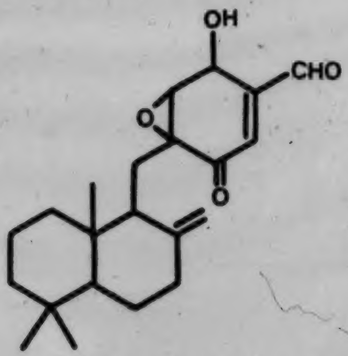
compatible with these observations.

During our investigation of compound 62 a number of trivial chemical transformations were performed to aid in the structural elucidation. The diacetylated compound 63 was particularly useful and has been discussed above.

Compound 62 was oxidised with CrO_3-pyridine complex in dichloromethane to give two discernable products. They were difficult to separate by ptlc, the best result being obtained by eluting twice with 30% petroleum ether in diethyl ether. Allylic oxidation of compound 62 with MnO_2 produced similar results. The main product formed was the



74



75

diketo derivative (74). The hrms of compound 74 indicates the parent ion, m/e 358 ($C_{22}H_{30}O_4$) and the expected base peak at m/e 137. The ir spectrum shows an hydroxyl absorption band (3440 cm^{-1}) and has a new absorption band in the carbonyl region at 1710 cm^{-1} which appears as a shoulder on the high intensity absorption band at 1687 cm^{-1}.

In the ^1H nmr of compound 74 the 3'-H signal moved downfield to 6.65 ppm (with respect to its chemical shift in compound 62) and appeared as a triplet (J = 2Hz) coupled to the methylene hydrogens of the 1° alcohol. The epoxide hydrogen (6'-H) appears as a sharp singlet at 3.75 ppm.

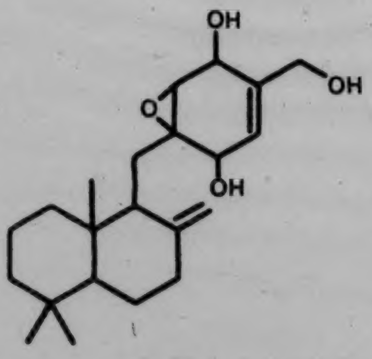
The diketo alcohol (74) was acetylated (acetic anhydride/pyridine) to give the mono-acetylated derivative (74a). The ir spectrum of compound 74a no longer has hydroxyl absorption but does have a new absorption band at 1750 cm^{-1} for the ester carbonyl. The ^1H nmr spectrum of compound 74a shows the signals for the C-4' methylene downfield ca. 0.3 ppm (with respect to the chemical shift in the ^1H nmr spectrum of compound 74) as expected and a new methyl singlet at 2.13 ppm. The hrms of compound 74a has a parent peak at m/e 400 ($C_{24}H_{32}O_5$) consistent with monoacetylation.

The second product (75) obtained from the oxidation of compound 62 has the same molecular formula as compound 74. The spectrum of compound 75 displays a one hydrogen singlet at 9.72 ppm which indicates that the primary alcohol of compound 62 has been oxidised to an aldehyde in compound 75. The sp^2 hydrogen at carbon-3' (6.48 ppm) is a doublet (J = 1Hz).

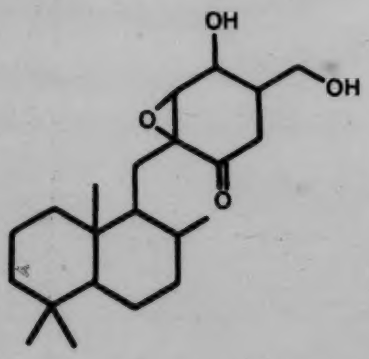
Compound 62 was reduced with $LiAlH_4$ in ether at room temperature to give compound 76. The product (76) was separated by ptlc. The ir spectrum has a large absorption band at 3380 cm^{-1} (O-H) and shows no significant absorption bands ca. 1700 cm^{-1} for a carbonyl functionality.

In the ^1H nmr spectrum of compound 76, the exo methylene hydrogens appear at 4.84 and 4.64 ppm. An sp^2 hydrogen appears upfield at 5.51 ppm and the H-6' signal which appears as a doublet (J = 3.5Hz) resonates at 3.53 ppm. The alcoholic methine hydrogens appear as one hydrogen multiplets at 4.81 and 4.43 ppm while the primary alcohol hydrogens appear as part of a ca. four hydrogen broad multiplet at 4.22 ppm.

The highest mass ion present in the hrms of compound 76 is at m/e 344 (M^+ - H_2O).



76



77

Compound 62 was hydrogenated (Pd/C, H_2 (1 atm)) producing the tetrahydro derivative 77. The hrms of compound 77 shows the parent peak at m/e 364 (1%) and the base peak at m/e 123.

The ir spectrum indicates that the carbonyl is no longer conjugated, the absorption band occurring at 1710 cm^{-1}. The ^1H nmr spectrum of compound 77 displays a broad multiplet at 4.18 ppm assignable to the methine-5', a two hydrogen broad multiplet at 3.72 (CH_2-4') and an epoxide hydrogen (H-6') which appears as a doublet (J = 3Hz) at 3.18 ppm. All these signals are shifted upfield with respect to the spectrum of compound 62 and lack the exocyclic methylene proton signals. The methyl signals of 77 coincide at 0.85-0.81 ppm.

As stated at the beginning of this section compound 62 was isolated specifically because of its apparent antifungal activity when fractions containing it were tested against C. albicans. No antifungal activity was reported for the similar compounds mentioned above. Epoxydon was shown to inhibit in vitro mitosis of mouse tumours[78] and is phytotoxic[79].

Compound 62 was tested for activity against C. ulmi. C. ulmi was grown on agar plates until it was established. Paper discs (ca. 6 mm diameter) soaked in methanol solutions of compound 62 were placed at least 15 mm away from the growing margin of the fungus. The lowest concentration of compound is equivalent to approximately 24 μg of compound 62 per disc. Table 5 gives the results obtained. As can be seen, significant inhibition still occurs at fairly low concentration. This compares very favourably to similar tests carried out on hyalodendrin (30) and nystatin[81], the former having been suggested as being a possible agent for combating Dutch Elm Disease.

Table 5

Bioassay of Compound 62 Against C. ulmi

Concentration* (mg/ml)	Inhibition Zone (mm)* Trial 1	Trial 2
9.6	6	3
4.8	5	6
2.4	5	6
1.2	9	5
0.6	5	4
0.3	5	3
blank (MeOH)	0	0

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*The discs were dipped twice into the MeOH solution.

*The inhibition zone was measured from fungus to the edge of the disc after the fungus had grown to the edge of the "blank" discs.

Part II Metabolites from Gremmeniella abietina

The strain of G. abietina studied was the so called virulent strain which was first detected in New York State in 1975. It is one of several strains available to us. Eventual comparison of the metabolites of each strain may give an indication of the presence of chemical pathogen(s) and the relationship between these and the level of virulence.

Stock cultures of G. abietina were maintained on agar slants, then transferred to agar plates which were then used to inoculate 500 mL shake cultures. The shake cultures provided inoculum to begin 10 L fermentations. In an alternative method the shake cultures were bypassed and the fermentor growths were inoculated directly from agar plates.

The medium used to grow the inoculum was a mixture of 1% glucose, 20% filtered V8 juice and water[6]. On agar plates this medium proved to be far superior to potato dextrose. The latter apparently does not provide the correct nutrients since the fungus does not grow more than one or two millimetres past the site of inoculation.

The concentration of V8 juice in the fermentation medium was reduced to 10% because growth and stagnation of the fungus using 20% V8 juice was very rapid and provided only a small quantity of metabolites when extracted with ethyl acetate.

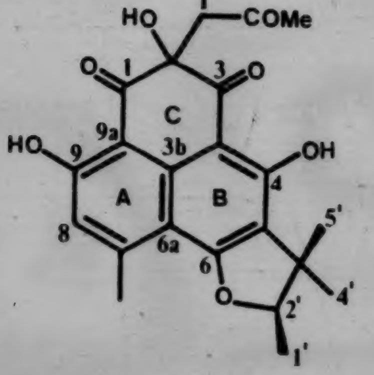
The fermentation was allowed to proceed for 11 days (10% V8 juice, 1% glucose) producing a brilliant dark green growth with a syrupy consistency. The dark green mycelium was separated from the medium which was extracted with ethyl acetate. The broth changed colour from green to rusty red during the process of extraction. The green colour partially returned when the medium was filtered through Celite after one EtOAc extraction and became orange again when it was stirred with a further portion of EtOAc. Thus it was with some trepidation that we proceeded with the purification of compounds realising that at least one compound was apparently quite unstable. The isolation of compounds 78 and 85 provided some justification for our concerns.

16. The Acetone Adduct of Atrovenetinone (78)

Compound 78 was isolated after chromatography of the crude metabolites from two separate 10 L fermentations of G. abietina. It is visible as a yellow spot on the tlc (R_f 0.7 in 10% methanol/chloroform) before and after treatment of the tlc with H_2SO_4/vanillin followed by heating.

The hrms spectrum of compound 78 reveals a parent ion at m/e 398 (45%) which has the formula $C_{22}H_{22}O_7$. The base peak appears at m/e 313 ($M^+ - C_4H_5O_2$). The ir spectrum has an absorption band at 3400 cm^{-1} (OH) and a medium intensity absorption band at 1710 cm^{-1} with a shoulder at ca. 1730 cm^{-1} as well as two strong absorption bands at 1635 and 1605 cm^{-1}.

The ^1H nmr spectrum gave us the first firm idea concerning a partial structure of compound 78. The spectrum displays methyl singlets at 1.30 and 1.52 ppm, a methyl doublet at 1.46 ppm (J = 6.5 Hz) coupled to a methine quartet at 4.64 ppm, an aromatic methyl at 2.78 ppm (doublet, J = 1Hz) coupled to an aromatic hydrogen at 6.76 ppm and two strongly hydrogen bonded OH hydrogens at 13.32 and 12.80 ppm. The ^1H nmr spectrum of compound 78 bears a strong resemblance to the ^1H nmr spectrum of compound (79) which had been isolated and identified by Dr. Y. Hoyano in these laboratories*. Since all the signals present in the anhydride 79 are present in compound 78 it was assumed to be identical with the anhydride with respect to the A and B rings; the differences in structure arising in ring C. The ^{1}H nmr spectrum of compound 78 had three other signals *viz* a broad one hydrogen signal at 3.70 ppm, a methylene singlet at 3.30 ppm and a methyl ketone singlet at 2.23 ppm.



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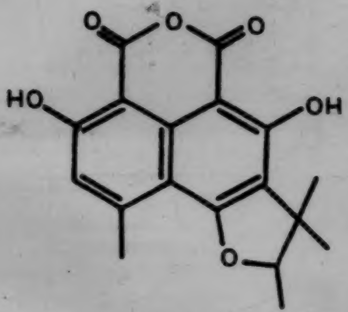
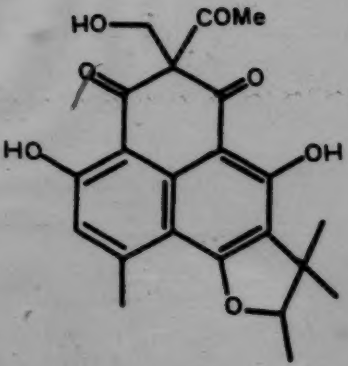


79

On the basis of this information two possible structures were proposed, *i.e.* those shown as 78 and 80. The structure 78 was assigned



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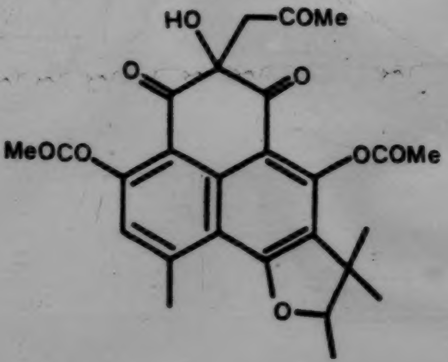

81

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*Dr. Hoyano has isolated compound 79 and several related compounds from a different, less virulent strain of the fungus *G. abietina*. The main secondary metabolites isolated from both strains of *G. abietina* have been shown to be remarkably similar, if not identical, as might be expected. I wish to take this opportunity to thank Dr. Hoyano for the free exchange of information pertinent to this project.

to the compound on the basis of its ^{13}C nmr spectrum. Carbons directly attached to oxygen have a reasonably distinctive chemical shift, i.e. 67-85 ppm for quaternary carbons and 40-70 ppm for secondary carbons. The substitution level of the carbons can be assigned by an off-resonance decoupled carbon spectrum. The four extra carbons in compound 78 w.r.t. the anhydride (79) were readily visible by comparison of the ^{13}C nmr spectra (see Table 7) and could be assigned by their multiplicities or chemical shift, e.g., methyl carbonyl (206.4 ppm). The quaternary carbon has a signal at 77.7 ppm which must mean that it bears an oxygen substituent, as depicted in structure 78 and not in structure 80. The chemical shift of the methylene (52.2 ppm) is not quite as definitive occurring in the chemical shift overlap region for carbon substituted methylenes and those with an oxygen substituent.

Acetylation of compound 78 provided only weak indirect evidence that the alcohol was tertiary since it resisted acetylation under normal conditions viz acetic anhydride and pyridine in dichloromethane. The acetylation procedure gave the diacetate (81) in which the phenolic functionalities have been acetylated. The hrms of the diacetate (81) has the expected parent ion at m/e 482 ($C_{26}H_{26}O_9$, 37%). The ir spectrum retains a broad absorption band at 3440 cm^{-1}, though less intense, but the acetyl carbonyl absorption band does appear at 1755 cm^{-1}. Comparison of the ^{1}H nmr spectra of compound 81 with compound 78 shows that the diacetate (81) spectrum has two new methyl singlets at 2.28 and 2.25 ppm, lacks the phenolic hydrogens, the tertiary alcohol signal moves upfield to 1.34 ppm and the methylene appears as an AB quartet (3.02 ppm, ^{2}J = 15Hz).

In principle it is possible to form four isomeric diacetates from compound 78 which arise through the tautomerisation of phenols and aromatic carbonyls. We propose the structure shown in 81 on steric grounds and the greater aromatic stability that can be ascribed to the retention of the naphthalene moiety. However a more rigorous investigation of the compound's structure would be necessary to remove all doubt concerning its precise structure. In fact, this question of tautomerisation arises in all the structures of this group and it is possible that the structures drawn for the compounds are in fact only representative of those actually present.

Throughout the work on compound 78 all the nmr spectra show signals which appear as pairs as if there were two diastereomeric isomers or slowly equilibrating tautomers present in the sample. Spin decoupling experiments or measurement of the ^1H nmr spectrum at higher temperatures did not collapse the pairs indicating the presence of diastereomers.

Generally, compounds from natural sources occur only as one enantiomer from a particular source. This led us to suspect that some kind of equilibration was occurring during isolation or that compound 78 was an artifact produced during isolation. An obvious "acetone" portion is present in the molecule. This is unusual in natural compounds and thus we suspected that compound 78 was formed by aldol condensation of acetone with a carbonyl in the 2-position. That compound 78 was indeed an artifact was confirmed in several ways. First, when acetone was rigourously excluded from all phases of the isolation, compound 78 was not detected. Secondly, we were able to synthesise compound 78 by the reaction of compound 85 (a 1,2,3-trione also isolated from the fungus) by stirring compound 85 at room temperature with acetone in the presence of a catalytic amount of acetic acid. A similar ninhydrin reaction will be discussed in the section concerning compound 85.

17. The Naphthalic Anhydride (4,7-Dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho[1,2-b]furan-5,6-dicarboxylic anhydride (79))

Compound 79 was isolated after flash chromatography[84] and Sephadex LH-20 separation was performed on the fungal medium extract. It is relatively non-polar (R_f 0.9 in $CHCl_3$:MeOH:AcOH (94:5:1) on tlc) and appears as a bright fluorescent spot on tlc under uv light. It is a white crystalline solid with a melting point of 252-54°C.

The hrms of compound 79 shows a molecular ion at m/e 328 ($C_{18}H_{16}O_6$) and a base peak at m/e 313 ($M^+ - CH_3$). In one case, at a slightly higher probe temperature (ca. 200°C instead of ca. 150°C) the mass spectrum changes to show a base peak at m/e 343, 15 mass units (CH_3) above the molecular weight. This can be explained as being due to an ion-molecule reaction in which a methyl group is transferred from an unionised molecule (probably the 1'-methyl) to the ether oxygen of an M^+ ion to form a methyl oxonium ion (i.e. m/e 343). However, we could not readily find examples of methyl transfer by gas phase ion-molecule reaction in normal low pressure mass spectrometry, but several cases of apparent methyl transfers have been reported resulting from an intermolecular thermal methyl transfer, occurring in the mass spectrometer prior to ionisation[85]. This thermal methyl transfer is found to be independent of sample pressure but related to ionisation temperature, occurring with compounds of low volatility.

Since the $M^+ + CH_3$ ion was also observed in the case of the two monomethylated anhydride derivatives (82 and 84) this mass spectral behaviour was investigated more closely. It was found (for the anhydride

79) that the rate of rise and fall of the total ion current corresponded to the rise and fall of the intensity of the M^+ and $M^+ - CH_3$ as the spectrometer probe was gradually heated. No $(M + CH_3)^+$ was observed and as there were no disparities in the ion currents the likelihood of an ion-molecule reaction is reduced.

The idea of a thermal methyl transfer also did not find support since the observation of the mass spectrum at varying temperatures (100°-250°C, probe temperature) showed no ion at $(M + CH_3)^+$ at the higher temperature, instead it appeared unexpectedly at the lowest temperature scan in one of the duplicate experiments. These observations remain unexplained.

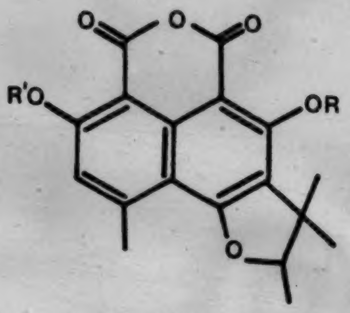
The ^1H nmr spectrum of compound 79 shows a methine quartet at 4.72 ppm (J = 6.5Hz) coupled to a methyl doublet at 1.50 ppm. The chemical shift of the methine is indicative of an aromatic ether substituent on the methine and the absence of further coupling is consistent with an adjacent quaternary carbon (4'). The gem-dimethyl group appears as two methyl singlets at 1.32 and 1.55 ppm. These two could be distinguished by an nOe experiment.

The aromatic hydrogen appears as a quartet (J = 1Hz) at 6.86 ppm coupled to the adjacent aromatic methyl group appearing at 2.82 ppm. The respective couplings were confirmed by spin decoupling experiments. The two hydrogen bonded phenol protons appeared at 11.42 and 11.64 ppm.

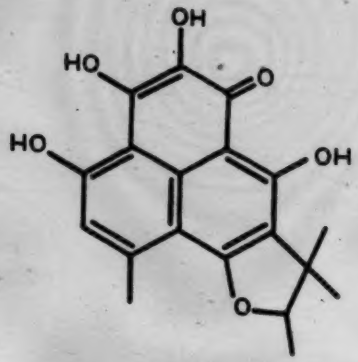
The ir spectrum of compound 79 shows two strong carbonyl absorption bands at 1670 and 1615 cm^{-1} which are remarkably low values for an anhydride. This is presumably due to strong hydrogen bonding between the carbonyls and the phenolic hydroxyls.

This data was identical with that shown by the naphthalic anhydride (79) as provided by Dr. Hoyano, with the exception that she did not observe the $(M + CH_3)^+$ peak in the mass spectrum. Dr. Hoyano's sample had previously been compared with an authentic sample provided by Professor Leo Vining.

The anhydride (79) was methylated by the slow addition of an ether solution of diazomethane to a stirred solution of compound 79 at room temperature. Diazomethane addition was stopped when a tlc of the reaction mixture showed that most of the starting material had reacted. The monomethyl compound (82) was isolated by ptlc as the major product.



82 R=H, R'=Me
84 R=Me, R'=H



83

Hrms of compound 82 confirms that monomethylation had occurred. The parent peak at m/e 342 (42%) which has the formula $C_{19}H_{18}O_6$, and a base peak at m/e 327 $(M^+ - CH_3)$ were observed. Again the issue is confused by a peak at m/e 357, $(M + CH_3)^+$. The cims clarifies the situation by showing peaks at m/e 702 $(2M + NH_4^+)$, 360 $(M + NH_4^+)$, 343 $(M + H^+)$, and 327 $(M^+ - 15)$ all masses expected for the monomethylated anhydride (82).

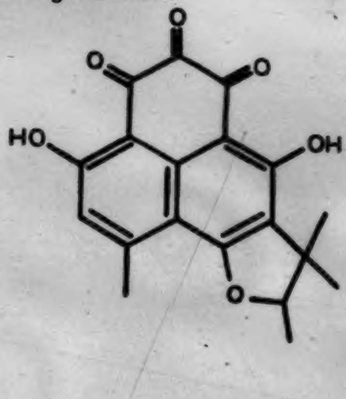
The ^1H nmr spectrum of compound 82 shows one phenolic hydrogen (12.31 ppm) and a methyl singlet at 4.12 ppm appropriate for an aromatic methyl ether. The ir spectrum has a strong absorption band at 1760 cm^{-1} as well as at 1650 cm^{-1} indicating that a carbonyl is no longer hydrogen bonded.

We propose that the phenol at carbon-9 is the one which is methylated as it is in a less sterically hindered environment than carbon-4. Barton et al[86] have reported the synthesis of both possible anhydrides (82 and 84) by degradation of two trimethyl ethers of atrovenetin (83)[87]. The authors report that the carbonyl absorption bands appear at 1750 and 1663 cm^{-1} for compound 82 and at 1747 and 1670 cm^{-1} for the alternate monomethyl ether (84).

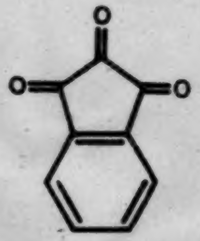
We believe that the minor compound isolated from the diazomethane reaction is the alternate monomethyl ether (84). Its ir spectrum has carbonyl absorption bands at 1750 and 1670 cm^{-1} as expected from Barton's work. Compound 84, although significantly less polar than its isomer (R_f 0.8 on tlc, 989:10:1 $CHCl_3$:MeOH:AcOH) has the same ion peaks in the (82) hrms. The ^{1}H nmr spectrum of 84 shows only slight changes in chemical shift when compared with that of 82. The enolic hydrogen was not observed in its usual position.

18. Atrovenetinone (85)

While extracts of the culture broth were being purified it was noticed that some of them caused a green discolouration of the skin even though the extract itself was a reddish brown. The compound that causes this discolouration was identified as atrovenetinone (85) in the following manner.



85



86

Compound 85 is a major component of the broth extract (R_f 0.75 in 5% MeOH in Chloroform on tlc; visualises yellow-green when sprayed with H_2SO_4/vanillin followed by heating). It was best purified by Sephadex LH-20 chromatography (MeOH), silica gel flash chromatography[84], using different proportions of CH_2Cl_2, diethyl ether, and methanol, followed by ptlc (1:19:80 AcOH/Et_2O/CH_2Cl_2). (In the choice of solvent it was important to eliminate the possibility of solvent addition to the extremely reactive central carbonyl.) This procedure provided pure samples of compound 85 although purification was hampered by solvent reactions and decomposition. Decomposition occurs rapidly when pure compound 85 is left to stand in the air, forming the anhydride (79) as the major compound.

Compound 85 is a dark burgundy crystalline solid which forms burgundy solutions in aprotic solvents (e.g. CH_2Cl_2, $CHCl_3$, Et_2O, benzene) but it changes colour when dissolved in protic solvents, e.g., with methanol compound 85 forms a green solution. The burgundy colour returns on evapouration of the methanol.

The hrms of compound 85 has six major ion peaks including m/e 340 (28%, $M^+(C_{19}H_{16}O_6)$), 312 (25%, M^+-CO), 297 (100%, M^+-CO-CH_3) and 269 (42%, M^+-2CO-CH_3). Its ir spectrum (CH_2Cl_2 cast) displays absorption bands at 3390 (OH), 1720 (weak), 1634 and 1605 (C=O) cm^{-1}.

The ^1H nmr spectrum of compound 85 was practically identical with that of the anhydride (79) suggesting that the changes in structure were in the C ring. These results can be explained if ring C contains a 1,2,3-trione.

The most significant difference in the ^1H nmr spectrum (DMSO-d_6) of compound 85 compared with the ^1H nmr spectrum ($CDCl_3$) of the anhydride

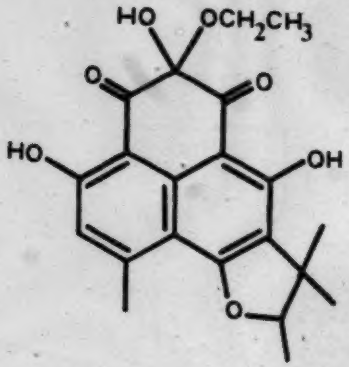
(79) is the chemical shift of the phenolic hydrogens, which may be a solvent induced shift.

As with other triones, e.g. ninhydrin (86), compound 85 is readily hydrated. Addition of H_2O to the ^1H nmr sample of 85 caused the appearance of a broad two hydrogen signal at 7.58 ppm with concomitant upfield movement of the phenolic hydrogens. In fact, compound 85 exists mainly in its hydrated form in solution as was shown by subsequent ^1H nmr spectra. In only one other case (see table 7, ^{13}C nmr spectrum in CD_2Cl_2) did we observe the signals of the free trione.

The chemical shifts found in a ^{13}C proton noise decoupled nmr spectrum of compound 85 are recorded in table 7.

Attempts were made to obtain the ^{13}C nmr spectrum of free trione 85 by recording the spectrum in DMSO-d_6 and CD_2Cl_2. In the DMSO-d_6 spectrum 85 exists in its hydrated form since both carbonyl signals (196, 198 ppm) and carbon-2 (88 ppm) appear at chemical shifts similar to those observed in the ^{13}C nmr spectrum of ninhydrin hydrate, (carbonyl 198 ppm, C-2 88 ppm[88, 89]). The CD_2Cl_2 ^{13}C nmr spectrum of 85 was more difficult to analyze because of the presence of ca. 20% of a similar compound. Despite this fact, it is obvious that the carbonyl signals appear upfield at 178 ppm. The highfield shift observed for the carbonyls is consistent with 85 in the free trione form; the more electronegative carbon-2 lessens the polarisation of the π-bond of the carbonyls thus causing an upfield shift. Similar chemical shifts have been observed for ninhydrin (86)[89].

In 1963 Vining[90] reported the structure of atrovenetinone (85), an oxidation product of atrovenetin (83). To our knowledge this is the first isolation of atrovenetinone from a natural source. The ethanolate of compound 85 was prepared by dissolving 85 in boiling ethanol. Upon standing greenish crystalline ethanolate 87 was formed. The melting point and ir spectrum of the ethanolate (87) were identical with those of an authentic sample (kindly provided by Dr. L.C. Vining).



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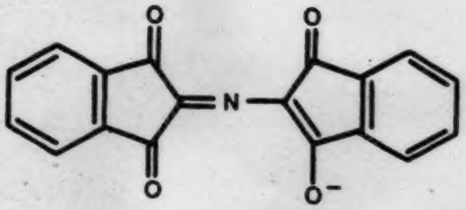
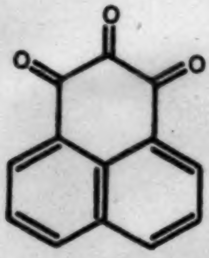


88

We have observed that the trione (85) reacts with amino acids (forming blue and green compounds), pyrrolidine, and ethanethiol but the nature of these products remains to be confirmed.

The ninhydrin reaction with amino acids to form a purple compound (Ruhemann's Purple[89], 88) is well known. Phenalene-1,2,3-trione (89) forms a light brown compound when reacted with amino acids[91]. The brown compound turns blue upon treatment with base. Thus it is not surprising that atrovenetinone reacts with amino acids to form blue compounds.



89

When solutions of atrovenetinone (85) are soaked into paper the formation of a greenish-blue colour is observed. Since the appearance of yellow-green to blue colours in areas of the pine tree is symptomatic of *G. abietina* infections, we propose ~~that~~ atrovenetinone (85), a metabolite of *G. abietina*, is responsible for the discolouration.

The reaction of atrovenetinone (85) with ethanethiol was monitored by ^{1}H nmr. These spectra indicate that there is a discrete addition of one ethanethiol molecule prior to the addition of the second ethanethiol. A spectrum recorded 30 minutes after addition shows an aromatic hydrogen as a small singlet at 6.72 ppm (*ca*. 20% reaction). This signal disappears with time and is absent in the spectrum of compound 90 being replaced by a singlet at 6.86 ppm. Table 6 lists the chemical shifts of atrovenetinone (85) (as its hydrate) and the spectrum of the ethanethiol addition product 90 formed after standing at room temperature for 18 hours.

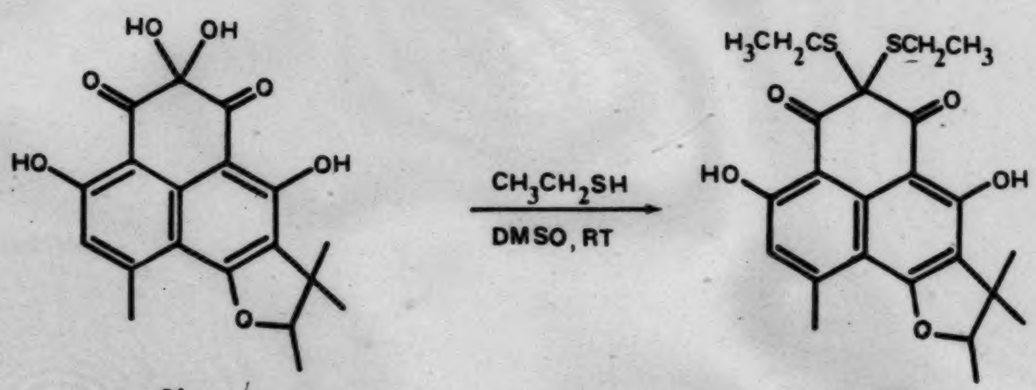
As mentioned earlier, an acetone-atrovenetinone adduct (78) was isolated as an artifact from the medium extract. Formation of adduct 78 occurs readily because of the reactivity of the central carbonyl of the 1,2,3-trione system.

The reactivity of the trione system was demonstrated in a model reaction. Ninhydrin (86) was treated with acetic acid in acetone. The white crystalline product (92) showed a parent ion at m/e 218 (13%) in hrms, absorption bands at 3320 (OH), 1745 (CO) and 1708 cm^{-1} (CO) in the ir spectrum and signals at 7.94 ppm (4 ArH), 3.76 ppm (OH) 3.31 ppm (CH_2) and 2.12 ppm (CH_3) in the ^{1}H nmr spectrum. The data is consistent with the adduct (92) as shown.

Table 6

^1H nmr Spectrum Chemical Shifts of the Reaction of Ethane Thiol and Atrovenetinone hydrate (91)

Hydrogen		Time (hrs)	
		0	18
4-OH	(s)	13.72	---
9-OH	(s)	12.95	---
2-OH	(s)	7.50	---
2-OH	(s)	7.45	---
S-C$\underline{H_2}$CH$_3$	(q)	---	2.70
S-CH$_2$C$\underline{H_3}$	(t)	---	1.25
8-H	(q)	6.88	6.80
2'-H	(q)	4.73	4.67
7-CH$_3$	(d)	2.75	2.76
1'-CH$_3$	(d)	1.44	1.44
4'-CH$_3$	(s)	1.48	1.50
5'-CH$_3$	(s)	1.25	1.26



91

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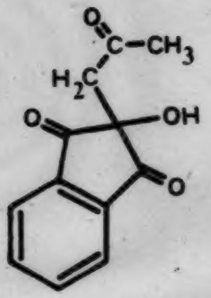

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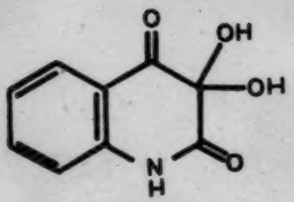

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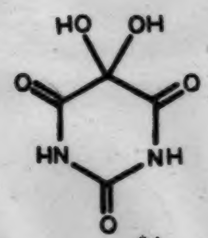
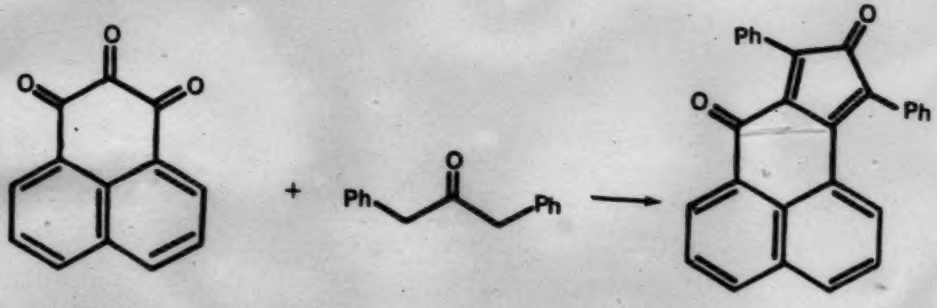


94

Over the years the 1,2,3-trione functionality has elicited substantial interest from chemists, particularly ninhydrin (86) and its reaction with various amines[92, 93]. Recently Schonberg and Singer[93] reviewed many of the reactions of this functionality including those of phenalene-1,2,3-trione(89), chinisatin hydrate (93) and alloxanhydrate (94). Many of the reactions involve addition to the central carbonyl but additions to the other carbonyls and cyclisations involving two adjacent carbonyls e.g. (95) have been observed.



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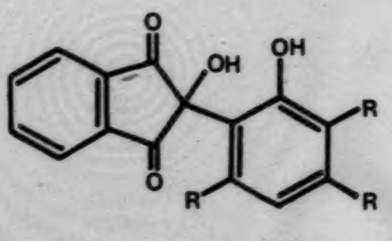
Since our initial interest in G. abietina lay in the fact that it was a pathogen of the Pinus species it was intriguing to find that the 1,2,3-trione system has been ascribed a wide variety of activities in differing biological systems, e.g., ninhydrin has been reported to bind to the thiol group in the active site of the protease papain causing it to be inhibited[94]. Clinical data suggests that some thiol inhibitors can cause regression in a variety of human cancers without injury to rapidly dividing normal cells of bone marrow or wounds[95]. Thus compounds with a 1,2,3-trione functionality are potential drugs for the regulation of the activity of nuclear protein thiol groups intimately concerned with gene control and neoplastic proliferation[96]. Both alloxan (94) and ninhydrin have been shown to inhibit glucose-induced insulin release[97]. It seems that the molecule size is important, i.e., it should be similar in size to glucose[98], thus phenalentrione-type compounds may not exhibit comparable reactivity. Finally, it has been shown that hydroxyphenylindandiones (96) synthesised from ninhydrin have an anti-inflammatory and analgesic effect[99].



96

In view of the many examples of biological activity of 1,2,3-trione systems it is not surprising that a fungus producing atrovenetinone would cause disruption of the host's growth. Possibly this arises by destroying essential amino acids or proteins or by blocking enzyme active sites. There are numerous questions which remain to be answered: e.g. What is the precise effect of atrovenetinone on Pinus metabolism?, Does G. abietina produce atrovenetinone directly or is it produced as a precursor which is oxidised externally?, How is the 1,2,3-trione reactivity modified by the strong peri hydrogen bonding of the phenols?

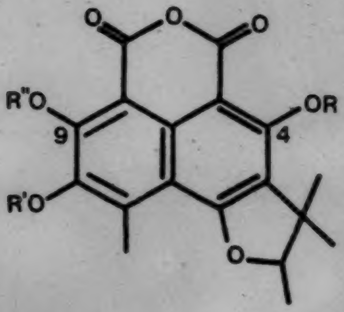
19. The 8-Hydroxynaphthalic Anhydride Methyl Ether (4,7,8-Trihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho[1,2-b] furan-5,6-dicarboxylic anhydride methyl ether) (97)

Investigation of the ethyl acetate extract of the mycelium of G. abietina by tlc showed the presence of generally the same compounds as those present in the medium. There were, however, some differences in the more polar compounds. Thus the fifth fraction from a Sephadex LH-20 column (CH_3OH) which appeared as a brown streak on tlc (from origin to R_f0.45 1:19 CH_3OH, $CHCl_3$ (AcOH)) was methylated with diazomethane in ether. The resulting mixture was purified by repeated ptlc producing two new compounds: the methyl ether (82) of the naphthalic anhydride (79) (compound 82 has been chemically correlated with anhydride 79 by methylation) and a bright yellow fluorescing (tlc, uv light) compound, 97.

The hrms of compound 97, m/e 358 ($C_{19}H_{18}O_7$, 76%) and m/e 343 (100%, M^+-CH_3) reveals that it contains one more oxygen than the naphthalic anhydride, 79.

The ^1H nmr spectrum (CD_2Cl_2) of compound 97 has the typical methyl group pattern and methine quartet of the atrovenetinone-type compounds. The alkyl methyl groups appear at 1.32, 1.50 and 1.56 ppm, the aromatic methyl at 2.83 ppm and the methine at 4.73 ppm. Besides the methyl ether singlet at 4.10 ppm, there is a broad one hydrogen signal at 6.47 ppm and one hydrogen bonded phenol at 11.94 ppm. There are no aromatic hydrogens present, thus the extra oxygen of compound 97 is most reasonably accommodated as a phenol at the carbon-8 position. The carbonyl stretching absorption bands in the ir spectrum of compound 97 are no longer at the extremely low frequencies, appearing instead at 1750 and 1685 cm^{-1}.

Thus methylation of the natural trihydroxyl compound (98) must have occurred at one of the phenolic positions C-4 or C-9. It is reasonable to assume that the methyl ether is at carbon-9 due to its less sterically crowded environment. However there are two pieces of evidence contradictory to this assignment. First, the lower frequency band observed in the ir spectrum of compound 97 is similar to the value observed for monomethyl ether 84 (methyl ether at carbon-4). Second, the ^1H nmr spectrum of compound 97 in pyridine showed only two significant changes[100]. The two labile hydrogens appear as a broad signal at 4.95 ppm (W 1/2 = 16Hz) and the aromatic methyl group moved downfield 0.26 ppm to 3.09 ppm. This data is consistent with an hydroxyl at carbon-8. The lack of significant solvent shift of the methoxyl and *gem*-dimethyl signals of 97 can be reasonably explained if the methyl ether is at carbon-4 (99).



97 R,R' = H; R" = Me
98 R,R',R" = H
99 R = Me; R',R" = H
100 R,R',R" = Me

The structure of 97 was clarified by an ^{1}H nmr experiment. The methoxyl singlet at 4.10 was irradiated in an effort to induce an NOE (nuclear Overhauser enhancement) of signals due to hydrogens in close proximity to the methoxyl group. We expected to observe significant enhancement in the *gem* dimethyl signals if the methoxyl group was at the carbon-4. No NOE is in fact observed, thus supporting the postulate that the methoxyl is at carbon-9.

The ^{13}C nmr spectrum of compound 97 verified the structural assignment. Comparison of the ^{13}C nmr of compound 97 with that of related compounds clearly showed the presence of the hydroxyl at carbon-8: by the absence of the higher intensity unsubstituted aromatic carbon and the presence of another signal in the 150 ppm region; and by the large upfield movement of the aromatic methyl group to 15.1 ppm as is expected for a methyl group *ortho* to a phenol[101]. Table 7 gives the ^{13}C nmr chemical shifts.

Compound 100, the trimethyl derivative of compound 97, has also been isolated by ptlc of diazomethane treated polar compounds.

The identification of compound 100 was based on hrms, ir and ^{1}H nmr spectroscopy. The ^{1}H nmr spectrum of compound 100 is typical of this group of compounds with aliphatic methyl groups at 1.32, 1.52 (doublet) and 1.56 ppm, an aromatic methyl singlet at 2.88 ppm and the methine quartet at 4.67 ppm. The spectrum displays signals for three methoxyl singlets at 4.13, 4.12 and 3.92 ppm.

The absorption bands in the ir spectrum of compound 100 for the aromatic anhydride carbonyls are at the more usual positions *viz.* 1760 and 1725 cm^{-1}. The hrms of compound 100 shows a parent ion ($C_{21}H_{22}O_7$) at m/e 386 (97%) and a base peak at m/e 371 ($M^{+}-CH_3$)

Table 7

^{13}C nmr Chemical Shifts of Some Atrovenetinone (85) Compounds*

Carbon No*	Compound 78 (CDCl$_3$)	 85 (DMSO-d$_6$)	 85 (CD$_2$Cl$_2$)	 79 (CDCl$_3$)	 82 (CD$_2$Cl$_2$)	 97 (Py-d$_5$)
3	199.8	197.6	179.8	165.4	165.3	166.7
2	77.7	87.9	177.4			
1	197.6	196.1	179.7	164.9	167.1	164.8
9a	110.0	108.9	111.1	108.6	109.8	108.4
9	165.7	164.7#	168.3	166.1	157.0	156.1
8	118.1	117.5	118.7#	117.4	112.6	148.2
7	149.7	148.0#	152.2	149.8	149.1	131.9
6a	103.0	101.9	108.3	93.6	93.8	94.1
6	166.3#	165.9#	168.3	166.2	166.1	163.1
5	119.1	118.0	119.5	119.2	119.3	120.5
4	166.5	165.3#	168.8	164.4	165.3	157.6
3a	105.8	104.8	110.0	97.3	101.1	107.1
3b	137.8	136.7	138.7	135.4	137.1	129.4
CH$_3$-7	24.3	23.5#	24.6#	23.6	24.3	15.1
1'	14.7#	14.2	14.7	14.5	14.7	14.5
2'	92.1#	91.2#	92.7#	92.2	92.4	91.7
3'	43.7	42.7	43.6	43.6	43.9	43.8
4'	25.8#	25.1	25.7#	25.7	25.8	25.6
5'	20.7	20.3	20.7	20.7	20.8	20.7
CH$_3$-O					57.0	61.7
1"	52.1#					
2"	206.3#					
3"	31.1#					

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*The assignment of the chemical shifts to particular carbons is based on benzene substituent effects applied to a naphthalene nucleus as well as other published observations[28] and must be regarded as tentative. This excludes the assignments for the anhydride (79) which have been assigned through specific proton decoupling of the coupled ^{13}C nmr spectrum[103].

*The numbering system is the same as shown for compound 78.

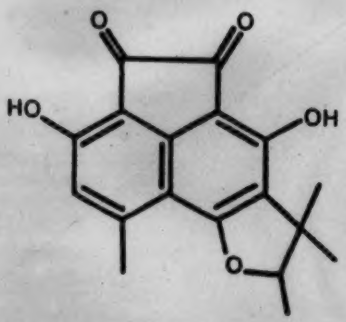
#These values represent an average of pairs of signals which exist due to diastereoisomerism, tautomerism or some other equilibrium effect.

consistent with the proposed structure.

The presence of compounds 97 and 100 as products of diazomethane reaction with the partially purified natural isolates suggests the presence of the parent trihydroxyl compound (98) in the mycelium of G. abietina.

20. 3,6-Dihydroxy-4,5-dioxo-1,7,7,8-tetramethyl-4,5,7,8-tetrahydroacenaphtho[5,4-b]furan (101)

The final compound of this series isolated from this strain of G. abietina was identified as the α-diketone (101) by comparison to an authentic sample (mp ^{1}H nmr, ir, hrms. Compound 101 was previously isolated and identified by Dr. Y. Hoyano).



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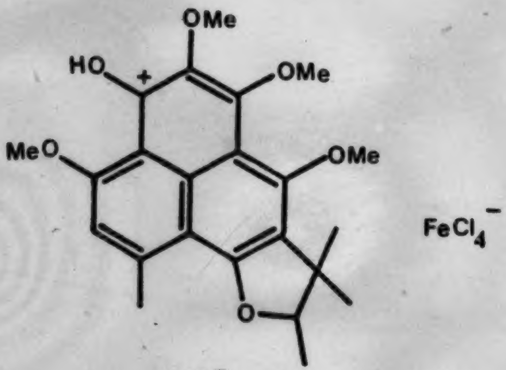

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Compound 101 is present in both medium and mycelium extracts. Upon double elution with 1:5:14 AcOH/petroleum-ether/$CHCl_3$ it moves to R_f 0.45. It is slightly more polar than atrovenetinone (85), and is visible as a distinct purple spot upon spraying with H_2SO_4/vanillin followed by heating. After purification in the usual way, compound 101 is obtained as bright red crystals (mp 200-202° C).

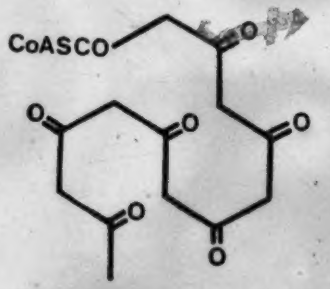
The hrms of compound 101 has three major mass peaks: m/e 312 (53%), M^+; m/e 297 (100%), M^+-CH_3 and m/e 269 (43%), M^+-CH_3-CO. The

^1H nmr spectrum of compound 101 has the expected signals, however the phenol hydrogens show as very broad singlets ($W1/2 = 16Hz$) indicating that they are no longer strongly hydrogen bonded. This absence of hydrogen bonding is also observed in the ir spectrum of 101 where the carbonyl absorption bands appear at 1710 and 1680 cm^{-1}.

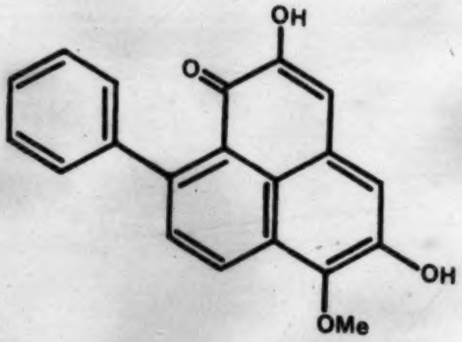
* * * * *

Compounds of the phenalenone class described above have been previously isolated from fungi, initially by Raistrick *et al* from *Penicillium herquei* in 1955[102].

The structure of the phenalenones was clarified in 1965 when Paul and Sim published the x-ray analysis of atrovenetin orange trimethyl ether ferrichloride [87](102). Biosynthetic studies have shown that the aromatic portion of the molecule is formed from a heptaketide chain folded in the manner shown (103) and that the dihydrofuran portion derives from mevalonate[103].



103



104

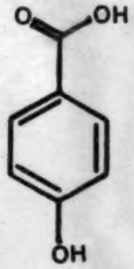
There is one other major class of naturally-occurring phenalenones as exemplified by the arylphenalenone, anigozanthin (104). These compounds have been isolated from plants of the *Haemodoraceae* family[104].

21. The Identification of Several Aromatic Hydroxy Acids

Repeated chromatography (ptlc) of the fifth Sephadex LH-20 fraction of several broth extracts of fermentations provided small amounts of components which proved to be a mixture of compounds. These compounds have relatively small molecular weights and are well known, thus it was possible to tentatively identify them from the spectra of the component mixtures.

Mixture 2-124S (R_f 0.45, 15% CH_3OH in $CHCl_3$ with one drop of acetic acid) contained p-hydroxybenzoic acid (105), coumaric acid (106) and 3-(p-hydroxyphenyl) propanoic acid (107) (dihydrocoumaric acid).

The identification was made on the basis of hrms and ^1H nmr spectroscopy. The hrms shows ion peaks at m/e 166 (25%), M^+(107); m/e 164 (71%), M^+(106); m/e 138 (84%), M^+(105); m/e 121 (100%), M^+(105)-OH; m/e 107 (86%), M^+(107)-$C_2H_3O_2$. Cims shows two peaks at m/e 184 and 182 corresponding to M(107) + NH_4^+ and M (106) + NH_4^+ respectively (100% and 99%).



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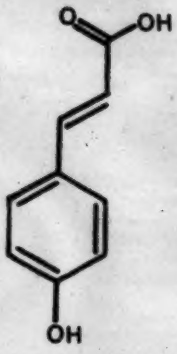

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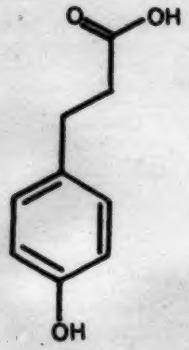
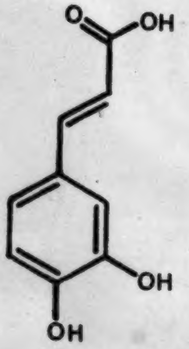


107

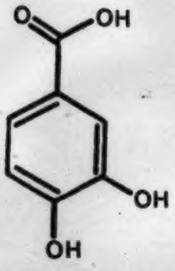
The peaks in the ^1H nmr spectrum of the mixture could be differentiated through their relative intensities and the chemical shifts of the different compounds compared well with published data[105].

Authentic samples of p-hydroxybenzoic acid and coumaric acid co-spotted on tlc eluted to give only one discernable spot. When authentic compounds 105 and 106 were co-applied on tlc with the isolated mixture, they eluted with the same R_f.

In a similar way we were able to tentatively identify the two compounds in mixture 2-124V as caffeic acid (108) and protocatechuic acid (109). Proton spin decoupling experiments (400 MHz) provided additional supportive evidence. The trans double bond hydrogens of compound 108 appear at 6.22 and 7.58 ppm (doublets, J = 16Hz); the aromatic hydrogens appear at 6.84 ppm (d, 8Hz), 6.96 ppm (dd,8,2 Hz) and 7.08 ppm (d,2Hz). The three aromatic hydrogens of compound 109 appear at 6.87 ppm (d,8Hz), 7.48 ppm (d,2Hz), 7.52 ppm (dd,8,2Hz).

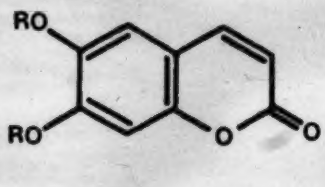


108



109

Mixture 2-124U contained mainly compound 110. Cims has an ion peak (100%) at 196 ($M+NH_4^+$) and another at m/e 179 ($M+H^+$). These are consistent with a molecular formula $C_9H_6O_4$. The ^1H nmr spectrum of



110 R = H
110a R = Me

compound 110 consists of two doublets (9Hz) at 6.22 and 7.60 ppm due to the hydrogens of a cis double bond and two one hydrogen singlets at 6.83 and 6.90 ppm for the aromatic hydrogens. The fact that the hydrogen signals are clear singlets indicates that they are para to each other.

Formation of the dimethyl derivative of compound 110 with diazomethane gave compound 110a. The ^{1}H nmr spectrum of compound 110a shows two one hydrogen doublets at 7.99 and 6.32 ppm, aromatic hydrogen singlets at 7.29 and 7.11 ppm, and aromatic methoxyl singlets at 3.89 and 3.83 ppm. The data agrees with that for esculetin, a well known compound. On this basis we tentatively assign the structure of compound 110 to be that shown.

These types of compounds are ubiquitous in nature and are usually products of shikimate metabolism. They often exhibit biological activity[36].

CONCLUSION

Conclusion

The studies reported here have provided a number of metabolites of diverse structures of varying degrees of complexity, novelty, and biological activity.

Our investigation of the metabolites of *P. brevi-compactum* was directed towards the isolation of the *C. ulmi* toxin. The study proved to be somewhat inconclusive. *P. brevi-compactum* produces a series of sulphur-containing compounds suggesting that an *epi*dithiapiperazine-2,5-dione compound may be present in the fungus. This type of compound is known to be active *in vitro* against *C. ulmi*. However, we did not isolate the *epi*dithiapiperazinedione-type compounds. Our experience with this fungus will make any future effort to isolate and identify a *epi*dithiapiperazine-2,5-dione metabolite simpler. As mentioned previously, alteration of the growth conditions by changing to growth of the fungus on agar plates or perhaps harvesting the fungus earlier would be the initial direction of the extension of this work.

It has been interesting to observe the relationships between the metabolites of our particular strain of *P. brevi-compactum* and those reported by other workers. Besides isolating mycophenolic acid (1) and deoxypebrolide (8) which are known metabolites of the fungus, we isolated a series of piperazine-2,5-dione compounds, a moiety which has also been found by Birch (the brevianamides). There is a similarity in the shape of the bioactive drimene 62 to that of compactin 7, previously isolated by others from this fungus. Even though the biogenesis of compactin has been suggested to arise from a nonaketide precursor (heavily reduced) the possibility that it is of terpene-ketide origin (a demethylated drimane) now is suggested.

Most of the other metabolites isolated from P. brevi-compactum have been isolated from other fungi. This then must act as a caution to those wishing to use chemotaxonomy in the identification of fungi and indicates that a spectrum of metabolites or metabolite types is necessary to provide a useful taxonomical key.

The metabolites of G. abietina remain under investigation. Through our initial work we have been able to isolate phenalenone-derived compounds which form a significant portion of the secondary metabolites of G. abietina. The phenalene compounds produce a colorful array, the colors giving rise to the characteristic features diagnostic of G. abietina infections in Pinus species. Biological tests remain to be performed, especially on atrovenetinone (85), to ascertain if the pathogenicity of G. abietina can also be ascribed to the phenalenone compounds. Our current investigations are directed toward the separation and purification of the more polar metabolites of G. abietina. These are expected, from first indications, to be highly phenolic and could also play a role in G. abietina's toxicity to pines.

EXPERIMENTAL

Experimental

All solvents except diethyl ether were distilled prior to use. ASC quality anhydrous diethyl ether was used without purification Petroleum ether refers to Skelly Oil Company light petroleum (Skelly B), bp 62-70°C. Pyridine was distilled from CaH_2 and stored over molecular sieves, acetic anhydride was dried over P_2O_5 and distilled from NaOAc.

Analytical thin layer chromatography (tlc) was carried out on glass microscope slides (75 x 25 or 75 x 50 mm) coated (ca. 0.3 mm) with silica gel G (W. Merck, Darmstadt or Terochem, Edmonton) containing 1% electronic phosphor (General Electric, Cleveland). Preparative thin layer chromatography (ptlc) was carried out on glass plates (20 x 20 cm) coated (0.6 mm) with the same adsorbent. Materials were detected by visualisation under an ultraviolet (uv) lamp (254 or 350 nm), or by spraying with a solution of vanillin (1%) in concentrated sulphuric acid. Careful charring on a hot plate followed by a brief cooling period produced the colours, observed as indicated in the text. Another visualisation agent used was 2% aqueous $NaIO_4$ (4 parts) and 1% $KMnO_4$ in 2% Na_2CO_3 (1 part) which indicated the components as white spots on a pink-purple background. Flash chromatography [84] was performed using Merck Silica Gel 60 (40-63 µm). Small scale column chromatography (<ca. 100 mg organic material) was performed with Silica Gel 60 (<0.08 mm) (Macherey Nagel, Duren) whereas larger scale chromatography was carried out with Silica Gel 60 (0.05-0.2 mm) (Machery Nagel, Duren).

Gel filtration chromatography was performed using Sephadex LH-20 (Pharmacia).

High resolution mass spectra (hrms) were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS 50 computer. Chemical ionisation mass spectra were recorded on an A.E.I. MS-9 mass spectrometer. Data is reported as m/e (molecular formula) relative intensity. Unless diagnostically significant, peaks with intensities less than 20% of the base peak are omitted. Infrared (ir) spectra were recorded on a Nicolet 7199 interferometer. Ultraviolet (uv) spectra were recorded on a Unicam SP 1700 ultraviolet spectrophotometer. ^{1}H nuclear magnetic resonance (^{1}H nmr) spectra were measured with a Varian HA-100 spectrometer, a Varian HA-100 spectrometer interfaced to a Digilab FTS/NMR-3 data system, a Bruker WH-200 spectrometer or a Bruker WH-400 spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C nmr) spectra were measured on a Bruker HFX-90 spectrometer interfaced to a Nicolet 1085 computer, a Varian HA-100 spectrometer interfaced to a Digilab FTS/NMR-3 data system, and a Bruker WH-200 spectrometer. All nuclear magnetic resonance measurements employed tetramethylsilane (TMS) as an internal standard and are reported in ppm downfield from TMS (δ). Optical rotations were measured on a Perkin Elmer Model 141 polarimeter and optical rotatory dispersion (ord) and circular dichroism (cd) measurements were made with a Durrum Jasco ORD/UV-5 (SS-20 modification) recording spectropolarimeter. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

Growth of Penicillium brevi-compactum and Extraction of Metabolites

Throughout this work, P. brevi-compactum was grown on Czapek SM liquid medium. This aqueous medium consists of the following nutrients per litre: sucrose, 30g; malt extract (Difco) 20g; $NaNO_3$, 3g; K_2HPO_4, 1g; KCl, 0.5g; $MgSO_4$, 0.5g; $FeSO_4$, 0.01g. Water used in all aspects of the culturing process was glass distilled. Stock cultures of P. brevi-compactum (C 662)* were maintained in Czapek SM medium agar slant tubes at 5°C. Large scale cultures were initiated in the following manner: small fragments of agar containing the mycelium were aseptically transferred to agar plates (10% mixed cereal, Pablum (Mead Johnson), 2% agar in water in 9 x 1 cm sterile petri dishes) and allowed to grow at room temperature until mycelial growth covered the entire surface of the agar plate. These growths were used to inoculate 500 mL Erlenmeyer flasks containing 250 mL of sterile** Czapek SM medium and gently shaken for ca. two days (the fungus was transferred by covering the agar plate with 10 mL sterile 0.01% sodium lauryl sulphate (or Tween 80) solution, scraping the fungus into the liquid and transferring it by sterile 10 mL pipette). Still cultures were grown in Fernbach flasks containing 1L sterile Czapek

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*Obtained from Dr. Y. Hiratsuka, Northern Forest Research Centre, Edmonton, Alberta

**Liquids were sterilised in an AMSCO American steriliser at 15 lb/sq. in. and 121°C from 15 mins. for 500 mL to 45 mins. for 10 L of medium for fermentation. Apparatus such as pipettes and funnels were wrapped in aluminium foil and autoclaved for 20 minutes.

SM medium for ca. 4 weeks after inoculation with 20 mL of the shake culture. Fermenations were carried out in a 10L New Brunswick Scientific MF-214 microferm laboratory fermentor. The fermentation was inoculated by addition of 500 mL of shake culture (2 x 250 mL) to 9.5 L of sterile Czapek SM medium containing 2 mL of polypropylene glycol 2000. The following standard conditions were used: 25°C, 200 r.p.m. agitation rate, and 2.5 L/min. air flow for ca. 8 days.

Several different methods of extraction and separation of the crude metabolites were employed. In the case of still cultures, the mycelial growth was separated from the broth by filtration through cotton wool. The mycelium was extracted by heating under reflux with ethyl acetate in a Soxhlet apparatus for 24 hours (yield ca. 0.11g per flask). The broth was stirred with ethyl acetate (1 part EtOAc to 3 parts broth) for 10 minutes and separated. This was repeated three times. The combined ethylacetate extracts were reduced *in vacuo* to ca. 700 mL, washed with brine and dried by vacuum filtration through anhydrous $MgSO_4$. The usual yield was 0.12g/L of broth. Initial separations of the crude extracts were made using gradient flash chromatography (solvent mixtures of increasing polarity: petroleum-ether to dichloromethane to ethyl acetate). Throughout this work silica gel columns were "washed out" with methanol to remove all but the extremely polar compounds.

The fermentation growths were processed in several different ways. In one case, the whole fermentor growth (mycelium and broth) was stirred with ethyl acetate using a Hershberg stirrer until new portions of ethyl acetate remained colourless. A colourless ethyl acetate extract indicated that the bulk of the organic soluble material had been extracted. The ethyl acetate was removed from the combined extracts *in vacuo* to provide the crude organic material. In the alternative procedure the broth was separated from the mycelium and the mycelium was extracted as before. The broth was reduced *in vacuo* to *ca*. 1 L, filtered through celite and extracted consecutively with 3 x 700 mL of toluene, ether, and *n*-butanol. These organic extracts were dried and evapourated *in vacuo*. Another preliminary separation procedure involved acid/base partitioning of an ethyl acetate extract in the following order: saturated $NaHCO_3$, 5% NaOH, and 1*N*HCl.

Generally, the crude organic extracts were separated on Sephadex LH-20 (100g, 65 cm x 2.8 cm) by elution with methanol at a flow rate which provided *ca*. 20 mL fractions every 40 minutes. The fractions were monitored by tlc and combined. Further separation of the combined fractions was performed using flash chromatography.

Growth of *Gremmeniella abietina* and Extraction of Metabolites

Stock cultures of *G. abietina* (previously also named Scleroderris largerbergii) (C 708)* were maintained on potato dextrose agar slant tubes at 5°C. *G. abietina* was transferred to agar plates (20% V8 juice (the V8 juice (Campbell Soup Co. Ltd., Toronto) was filtered through Celite prior to use), 1% glucose, 2% agar). The procedure involves addition of sterile 0.01% sodium lauryl sulphate solution to the slant tube, scraping off pieces of the fungus with a sterile pasteur pipette and pipetting the solution on to the agar plate. After 12 days the fungus was transferred to shake cultures (250 mL 20% V8 juice, 1% glucose medium in 500 mL Erlenmeyer flasks) in the same way as above. The culture was gently shaken for 16-25 days at 17°C. Two shake cultures were used to inoculate a 10 L fermentator (9.5 L, 10% V8 juice, 1% glucose containing 3 mL of polypropylene glycol). The following standard conditions were used: 18°C, 200 r.p.m. agitation rate, and 2.5 L/min air flow for _ca_. 11 days. Despite the addition of antifoaming agent the fermentation growth produced substantial amount of foam on the surface of the broth.

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*Obtained from Dr. Y. Hiratsuka, Northern Forest Research Centre, Edmonton, Alberta

The mycelium was separated from the broth by filtration through cotton wool. The dark green mycelium was extracted with ethyl acetate in a Soxhlet apparatus for 48 hours. The broth was extracted by stirring with aliquots of ethyl acetate (_ca_. 1/3 broth volume) for 10 mins/aliquot. This was repeated until no substantial colouration of the ethyl acetate was observed. The ethyl acetate extracts of either the mycelium or the broth were reduced _in vacuo_ to _ca_. 700 mL, washed with saturated NaCl solution, dried over anhydrous $MgSO_4$, vacuum filtered and the ethyl acetate was removed _in vacuo_.

In one case the inoculum for fermentation was prepared by blending the fungal growth from two agar plates with 400 mL sterile water for 15 sec. This inoculum was introduced directly into the 10 L fermentor.

One litre still cultures were inoculated by sterile transfer of 20 mL of inoculum obtained from shake cultures. After 40 days these were swirled vigourously and extracted after 80 days. A medium of 10% V8 juice and 1% glucose and a temperature of 17°C were used. The extraction procedure was identical with that used for the fermentations.

In all cases the crude extracts were dissolved in $\leq$20 mL of methanol, filtered through a cotton wool plug and applied onto a Sephadex LH-20 column (100g, 65 cm x 2.8 cm). The column was eluted with methanol at a flow rate which provided ca. 20 mL fractions when collected at 40 minute intervals. A typical total column yield from the broth extract of a 10 L fermentation was between 3.5 and 6 grams; the major portion (appearing in ca. tubes 1 to 16) being antifoam agent. The bulk of the organic compounds obtained from G. abietina appeared in ca. tubes 20 to 45, yielding ca. 1.5g of material. The larger fractions from the Sephadex LH-20 column (>0.15g) were generally further fractionated by silica gel flash chromatography using gradient elution with dichloromethane, ether and methanol mixtures of increasing polarity.

Isolation of Compounds 8 and 44

Mycelium extract (2.5g) of P. brevi-compactum was slurried with silica gel (5g) and methanol and the solvent carefully evapourated *in vacuo*. The resultant powder was applied to a flash column (5 cm diameter) and a gradient (petroleum ether-dichloromethane-ethyl acetate-methanol) elution was performed. Compounds 8 and 44 were obtained in one fraction (30% ethyl acetate in dichloromethane) (72 mg). Trituration with ether provided compound 44 (33 mg) as a solid. Compound 8 (13 mg) was isolated as a pure compound after ptlc of the ether soluble compounds (chloroform: methanol: acetic acid: 189:10:1).

tlc (compound 8): R_f 0.3 (chloroform:methanol:acetic acid, 94:5:1), pink spot

tlc (compound 44): R_f 0.5 (chloroform:methanol:acetic acid, 189:10:1), uv visible or with $KMnO_4$/$NaIO_4$.

1-Deoxypebrolide (8; (3aS,5R,5aS,6R,9aR,9bS)-6-acetoxymethyl-5-benzoxy-6,9a-dimethyl-3-oxododecahydronaphtho[1,2-c]furan)

Formula (MW): $C_{24}H_{30}O_6$ (414).

Mp: 164-166°C (Benzene/petroleum ether, white crystals).

$[\alpha]_D^{26}$: -49°C ($\underline{c}$ 0.62, $CHCl_3$).

ir ($CHCl_3$ cast): 2930, 2878, 2860, 1779, 1738, 1713, 1278, 1249, 1150, 1109, 1052, 716 cm^{-1}.

uv (MeOH) λmax (ε): 207 (10,700), 229 (14,500), 274 (1,656), 281 (1,348) nm.

^1H nmr ($CDCl_3$): 7.96 (2H, dd(8,2Hz), ArH); 7.46 (3H, m, ArH): 5.70 (1H, m (W1/2 = 9Hz), OCH); 4.38 (1H, d (9Hz), $\underline{H}$CHO; 4.18 (1H, dd (9,5Hz), $\underline{H}$CHO; 4.00 (1H,d (12Hz), $\underline{H}$CHO); 3.75 (1H,d(12Hz), $\underline{H}$CHO); 2.68 (1H,m), 2.55 (1H,m); 2.06 (3H,s,CH_3); 1.42 (3H,s,CH_3); 0.98 (3H,s,CH_3) ppm.

^{13}C nmr ($CDCl_3$): 177.2, 170.2, 165.9, (C=O); 132.3, (=CH); 130 (=C); 129.1, 127.9, (2 x =CH); 72.0, (OCH); 67.0, 66.9, (OCH_2); 48.9, 46.8, 42.2, 37.2, 36.6, 35.2, 34.2, 27.5, 20.3, 19.0, 17.6, 16.9 ppm.

hrms: m/e calcd for $C_{17}H_{25}O_5$ (M^+ - C_6H_5CO):309.1702;

found: 309.1699 (4%); 292 ($C_{17}H_{24}O_4$)6; 249 ($C_{15}H_{21}O_3$)31; 232 ($C_{15}H_{20}O_2$) 12; 105 (C_7H_5O)100; 77 (C_6H_5)28.

cims: 432(M + NH_4^+).

Asperphenamate (44; (S)-N-benzoylphenylalanine-(S)-2-benzamido-3-phenylpropyl ester)

Formula (MW): $C_{32}H_{30}N_2O_4$ (506).

Mp: 205-207°C (dichloromethane, white solid).

$[\alpha]_D^{26}$ (c 1.01, pyridine): -93.6°.

ir (nujol): 3300, 3060, 3030, 1750, 1740, 1638, 1530, 1220, 690 cm^{-1}.

^{1}H nmr (CDCl$_3$/DMSO-d$_6$): 8.52 (1H,d(7Hz),NH); 7.96 (1H,d(8Hz),NH); 7.78 (4H,m,ArH); 7.35 (6H,m,ArH); 7.23 (5H,s,ArH); 7.19 (5H,s,ArH); 4.77 (1H,td(8,7Hz)CH); 4.46 (1H,bs,CH); 4.40 (1H,m,HCH); 4.02 (1H,m,HCH); 3.24 (1H,s,HCH); 3.26 (1H,d(3.5Hz),HCH); 2.90 (2H,d(7Hz), CH$_2$) ppm.

^{13}C nmr (CDCl$_3$/DMSO-d$_6$ (3:1)): 171.7, 167.7, 167.2, 137.8, 137.1, 134.7, 133.9, 131.5, 131.1, 129.2, 128.4, 128.3, 128.1, 127.5, 127.4, 126.7, 126.4, 65.2, 54.9, 50.3, 37.0, 34.1 ppm.

hrms: m/e calcd. for $C_{25}H_{23}N_2O_4$ ($M^+ - C_7H_7$): 415.1657; found: 415.1653 (10%); 385($C_{25}H_{23}NO_3$)3; 294($C_{18}H_{16}NO_3$)6; 252($C_{16}H_{14}NO_2$)14; 224($C_{15}H_{14}NO$) 15; 146(C_9H_8NO)22; 105(C_7H_5O)100; 91(C_7H_7)14; 77(C_6H_5)34.

cims: 507(M + H$^+$).

14-Desacetyl-1-deoxypebrolide (8a; (3aS,5R,5aS,6R,9aR,9bS) -5-benzoxy-6,9a-dimethyl-6-hydroxymethyl-3-oxododecahydronaphtho [1,2-c]furan)

Compound 8 (3.5 mg) was stirred with aqueous methanol (1:1, 1mL) and triethylamine (5 drops) at room temperature (RT) for 24 hours. The solid product was recovered by evapouration of the liquids. Preparative thin layer chromatography on an analytical silica gel plate provided the deacetylated product 8a (1 mg).

Formula (MW): $C_{22}H_{28}O_5$ (372).

ir (CHCl$_3$ cast): 3480, 2930, 1774, 1710, 1280, 1150, 1110, 1050, 710 cm^{-1}.

^{1}H nmr (CDCl$_3$): 7.99(2H,dd(8,2Hz), ArH; 7.50(3H,m,ArH); 5.74(1H,m, W1/2 = 8Hz),OCH; 4.40(1H,d(10Hz),HCHO); 4.22(1H,dd(10,6Hz),HCHO);

3.70(1H,d(11.5Hz)HCHO); 3.15(1H,d(11.5Hz),HCHO); 2.64(1H,d(8Hz)CHC=O); 2.59(1H,bd(16Hz),HCH); 2.32(1H,dd(8,6Hz),CH); 2.04(1H,dd(16,5Hz),HCH; 1.66(1H,bs,W1/2 = 2Hz),CH); 1.42(3H,s,CH_3); 0.87(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{22}H_{28}O_5$: 372.1937; found 372.1947 (0.5%); 267($C_{15}H_{21}O_4$)15; 250($C_{15}H_{22}O_3$)19; 249($C_{15}H_{21}O_3$)21; 232($C_{15}H_{20}O_2$)10; 220($C_{14}H_{20}O_2$)50; 105(C_7H_5O)100; 77(C_6H_5)31.

tlc: R_f 0.65 (chloroform:methanol:acetic acid, 89:10:1) pink spot.

The europium shift ^1H nmr experiment was conducted in the following manner: Eu(fod)$_3$ (41 mg) was dissolved in $CDCl_3$ (2 mL) and 10 μL aliquots of the solution were added to the ^1H nmr tube containing compound 8a (1 mg), the ^1H nmr spectrum being recorded prior to each addition. A total of 11 additions were made. The results of this experiment are recorded in graphical form in Figure 2.

14-Desacetyl-6-debenzoyl-1-deoxypebrolide (12;6,9a-dimethyl-5-hydroxy-6-hydroxymethyl-3-oxododecahydronaphtho [1,2-c]furan)

Compound 8 (10 mg) was heated under reflux with 2N NaOH (10 mL) for 90 minutes. The reaction mixture was acidified (HCl), extracted with $CHCl_3$ and washed consecutively with saturated Na_2CO_3 H_2O and brine. This reaction gave a 9:5 mixture of the two isomers 12a:12b (by ^1H nmr spectroscopy).

Formula (MW): $C_{15}H_{24}O_4$ (268).

ir ($CHCl_3$ cast): 3480, 2930, 2870, 1765, 1260, 1160, 1040, 985 cm^{-1}.

^1H nmr ($CDCl_3$) (12a): 4.52(1H,ddd(3,3,2Hz)OCH); 4.26(1H,dd(8,7Hz),HCHO); 4.06(1H,dd(11,8Hz),HCHO); 3.75(1H,d(11Hz),HCHO); 3.22(1H,d(11Hz),HCHO); 2.81(1H,ddd(14,12.5,3.5Hz),CHC=O); 2.17(1H,ddd(12,3.5,3Hz),HCH);

1.93(1H,ddd(12.5,11,7Hz),CH); 1.59(1H,ddd(14,12,3Hz),$\underline{H}$CH); 1.31(3H,s,CH$_3$); 1.27(1H,d(2Hz),CH); 1.19(3H,s,CH$_3$) ppm.

hrms: m/e calcd. for $C_{15}H_{22}O_4$ ($M + H^+$): 269.1753; found 269.1757 (1%); 237($C_{14}H_{21}O_3$)29; 221($C_{13}H_{17}O_3$)13; 219($C_{14}H_{19}O_2$)59; 173($C_{13}H_{17}$)20; 127($C_6H_7O_3$)100; 109(C_8H_{13})42; 95(C_7H_{11})21; 81(C_6H_9)37; 55(C_4H_7)23.

cims: 286($M + NH_4^+$), 554 ($2M + NH_4^+$).

tlc: R_f 0.5 (chloroform:methanol:acetic acid, 89:10:1) pink spot.

Isolation of Compounds 14, 20, 34, 35, and 40

The broth of 10 L of fermentation culture of *P. brevi-compactum* was reduced in volume to 1L *in vacuo* and extracted 3 times with 700 mL portions of toluene. The combined toluene extracts were reduced *in vacuo* to *ca*. 200 mL from which 450 mg of compound **14** crystallised as a white solid. The solvent was removed from the mother liquor *in vacuo* providing a mixture (*ca*. 150 mg) which was chromatographed on a silica gel column (*ca*. 20g) by gradient elution with toluene/acetone. The fourth fraction (*ca*. 20% acetone/toluene) contained mostly compound **34**, the sixth (*ca*. 35% acetone/toluene) contained compound **40**, the ninth fraction (*ca*. 50% acetone/toluene) gave pure compound **20** (70 mg), and compound **35** (10 mg) crystallised from the final elution of the column with methanol.

Compounds **34** and **40** were purified by ptlc (double elution with toluene:acetone:acetic acid; 69:30:1) providing 70 mg of **34** and 2 mg of **40**.

tlc (compound **14**): R_f 0.45 (chloroform:methanol:acetic acid; 89:10:1), blue spot.

tlc (compound **20**): R_f 0.55 (chloroform:methanol:acetic acid; 89:10:1), yellow spot.

tlc (compound 34): R_f 0.85 (chloroform:methanol:acetic acid; 89:10:1) yellow spot.

tlc (compound 35: R_f 0.75 (chloroform:methanol:acetic acid; 89:10:1) light brown spot, uv visible.

tlc (compound 40): R_f 0.25 (chloroform:methanol:acetic acid; 189:10:1) light brown spot, uv visible.

Mycophenolic acid (14; 6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzylfuranyl)-4-methyl-4-hexenoic acid

Formula (MW): $C_{17}H_{20}O_6$(320).

Mp: 143-145°C (benzene/petroleum-ether, clear crystals).

Analysis calcd. for $C_{17}H_{20}O_6$: C, 63.74%; H, 6.29%;

found: C, 63.6%; H, 6.26%.

ir ($CHCl_3$ cast): 3426, 1730, 1705, 1620, 1075 cm^{-1}.

uv (MeOH) λmax (ε): 219(28500), 250(8480), 306(4140) nm.

^{1}H nmr ($CDCl_3$): 8.92(1H,bs,OH); 5.27(1H,tq(7,0.5Hz),=CH); 5.16(2H,s,CH_2O); 3.72(3H,s,OCH_3); 3.36(2H,bd(7Hz),CH_2); 2.18(4H,m,CH_2-CH_2); 2.12(3H,s,ArCH_3); 1.78(3H,d(0.5Hz),=CCH_3) ppm.

^{13}C nmr ($CDCl_3$): 175.1, 172.9, C=O; 163.8, 157.7, 145.5, 134.3, =C-; 123.4, CH; 122.5, 117.1, 101.6, =C-; 70.2, OCH_2; 61.2, OCH_3; 35.3, 33.3, 23.3, CH_2; 16.2, 11.3, CH_3 ppm.

hrms: m/e calcd. for $C_{17}H_{20}O_6$: 320.1260; found 320.1253 (39%); 302($C_{17}H_{18}O_5$)33; 261($C_{15}H_{17}O_4$)16; 247($C_{14}H_{15}O_4$)100; 245($C_{14}H_{13}O_4$)25; 229($C_{14}H_{13}O_3$)32; 219($C_{12}H_{11}O_4$)27; 207($C_{11}H_{11}O_4$)62; 159($C_{10}H_7O_2$)25.

N,N-dimethyl-3,6-dithiomethyl-3-(4' hydroxyphenyl) methylpiperazine-2,5-dione (20)

Formula (MW): $C_{15}H_{20}N_2O_3S_2$ (340).

Mp: 154.5-156°C (benzene/petroleum ether, yellowish crystals).

$[\alpha]_D^{26}$: -27.6° (c 0.642, $CHCl_3$).

ord (c 0.643, CH_3OH) 26°C: $[\phi]_{340}$ 0° (intersects), $[\phi]_{287}$ + 1590° (peak), $[\phi]_{266}$ 0° (intersects), $[\phi]_{244}$ - 5300° (trough), $[\phi]_{230}$ 0° (intersects).

ir ($CHCl_3$ cast): 3350, 2920, 1665, 1657, 1620, 1519, 1440, 1390, 1270, 1240 cm^{-1}.

uv (MeOH) λmax (ε): 220(14,200), 276(1,690) nm.

^1H nmr ($CDCl_3$): 6.91(2H,d(9Hz),ArH); 6.70(2H,d(9Hz),ArH); 4.17(1H, s,CH); 3.48(1H,d(14Hz),HCH; 3.30(3H,s,NCH_3); 3.08(1H,d(14Hz),HCH); 2.95(3H,s,NCH_3); 2.26(3H,s,SCH_3), 2.16(3H,s,SCH_3) ppm.

^{13}C nmr ($CDCl_3$): 165.6, 164.4, C=O; 156.4, ArC; 130.7, 2 x ArCH; 125.0, ArC; 115.6, 2 x ArCH; 75.7, C; 64.9, CH; 42.1, CH_2; 33.6, 30.6, NCH_3; 15.9, 13.6, SCH_3 ppm.

hrms: m/e calcd. for $C_{15}H_{20}N_2O_3S_2$: 340.0915; found 340.0912 (2.8%); 293($C_{14}H_{17}N_2O_3S$)62; 246($C_{13}H_{14}N_2O_3$)18; 233($C_8H_{13}N_2O_2S_2$)26; 218($C_{12}H_{14}N_2O_2$) 27; 107(C_7H_7O)100; 88(C_3H_6NS)17; 77(C_6H_5)29.

cims: 358(M + NH_4^+), 698(2M + NH_4^+).

N,N-dimethyl-3,6-dimethylthio-3-(4'-(3"-methyl-2"-butenoxy) phenyl) methylpiperazine-2,5-dione (34)

Formula (MW): $C_{20}H_{28}N_2O_3S_2$ (408).

Mp: 63-65°C (benzene/petroleum-ether, fatty yellow solid).

$[\alpha]_D^{26}$ = -39.5 (c 1.36, $CHCl_3$).

ord (c 1.41, MeOH), 26°C: $[\phi]_{285}$ + 1,160° (peak); $[\phi]_{252}$ 0° (intersects); $[\phi]_{220}$ - 2,170° (trough); $[\phi]_{200}$ 0° (intersects).

cd (c 0.010, MeOH), 26°C: $[\theta]_{247}$ + 2,910; $[\theta]_{235}$ 0 ; $[\theta]_{217}$ + 9,240.

ir ($CHCl_3$ cast): 2955, 2920, 2845, 1730 (w), 1665, 1460, 1440, 1385, 1240 cm^{-1}.

uv (meOH) λmax (ε): 205(shl, ca. 30,600), 226(33,300), 270(6,460) nm.

^1H nmr ($CDCl_3$): 7.00(2H,d(9Hz),ArH); 6.80(2H,d(9Hz),ArH); 5.50(1H,bt (7Hz),=CH); 4.48(2H,d(7Hz),OCH_2); 4.23(1H,s,CH); 3.58(1H,d(14Hz),$\underline{H}$CH); 3.26(3H,s,NCH_3); 3.09(1H,d,14Hz); 2.98(3H,s,NCH_3); 2.28(3H,s,SCH_3); 2.16 (3H,s,SCH_3); 1.81(3H,d(1Hz),=CCH_3); 1.76(3H,d(1Hz),=CCH_3) ppm.

^{13}C nmr ($CDCl_3$): 165.0, 164.5, 158.5, 138.1, 130.7(2); 126, 119.8, 114.8(2), 65.2, 64.9, 41.9, 33.5, 30.2, 25.8, 18.2, 16.2, 13.6 ppm.

hrms: m/e calcd. for $C_{19}H_{25}N_2O_3S$ (M^+ - SCH_3): 361.1586; found: 361.1573 (43%); 293($C_{14}H_{17}N_2O_3S$)43; 246($C_{13}H_{14}N_2O_3$)15; 245($C_{13}H_{13}N_2O_3$)39; 233($C_8H_{13}N_2O_2S_2$)63; 186($C_7H_{10}N_2O_2S$)31; 107(C_7H_7O)100; 88(C_3H_6NS)9.

cims: 426(M + NH_4^+).

3-Thiomethyl-3-(4'-(3"-methyl-2"-butenoxy) phenylmethyl) piperazine-2,5-dione (35)

Formula (MW): $C_{17}H_{22}N_2O_3S$ (334).

Mp: 205-207°C (dichloromethane, white solid).

$[\alpha]_D^{26}$ (c 0.020, $CHCl_3$) = +8.4.

cd (c 0.0080, MeOH), 26°C: $[\theta]_{225}$ 0 ; $[\theta]_{205}$ + 500.

ir (nujol): 3180, 3070, 2920, 2870, 1688, 1510, 1455, 1375, 1245 cm^{-1}.

uv (MeOH) λmax(ε): 207(14,000), 229(15,000), 277(4,000) nm.

^1H nmr (DMSO-d_6): 8.84(1H,s,NH); 8.01(1H,bs($W1/2$ = 5Hz),NH); 7.18 (2H,d(9Hz),ArH); 6.76(2H,d(9Hz)ArH); 5.58(1H,bt(6Hz),=CH); 4.43(2H,d(6Hz),

OCH_2); 3.79(1H,d(18Hz),$\underline{H}$CH); 3.41(1H,d(13Hz),$\underline{H}$CH); 3.30(1H,dd(18,3Hz), $\underline{H}$CH); 2.81(1H,d(13Hz),$\underline{H}$CH); 2.15(3H,s,SCH_3); 1.70(3H,s,=CCH_3); 1.66(3H, s,=CCH_3) ppm.

^{13}C nmr (DMSO-$\underline{d}_6$): 166.0, 164.5, C=O; 157.5, 136.7, =C; 131.7, 2 x ArCH; 127.3, ArC; 120.1, 114.1(2), =CH; 66.5, C; 64.3, OCH_2; 44.3, 41.6, CH_2; 25.3, 17.9, 12.6, CH_3 ppm.

hrms: m/e calcd. for $C_{17}H_{22}N_2O_3S$: 334.1352; found 334.1371 (1%); 266($C_{12}H_{14}N_2O_3S$)13; 219($C_{11}H_{11}N_2O_3$)62; 218($C_{11}H_{10}N_2O_3$)57; 175($C_{12}H_{15}O$)23; 160($C_5H_8N_2O_2S$)30; 159($C_5H_7N_2O_2S$)27; 107(C_7H_7O)100; 69(C_5H_9)66.

N,N-dimethyl-3-hydroxy-3-(4'-methoxyphenylmethyl) piperazine-2,5-dione (40)

Formula (MW): $C_{14}H_{18}N_2O_4$ (278).

Mp: 166-167°C (dichloromethane, clear crystals).

ir ($CHCl_3$ cast): 3360, 2935, 2840, 1670, 1605, 1515, 1455, 1395, 1300, 1240, 1180, 1155, 1120, 1095, 1025, 600, 570 cm^{-1}.

uv (MeOH) $\lambda max(\varepsilon)$: 213(6,600), 228(8,690); 276(1210) nm.

^1H nmr ($CDCl_3$): 6.94(2H,d(8Hz),ArH); 6.80(2H,d(8Hz),ArH); 4.42(1H, bs(W1/2 = 13Hz),OH); 3.78(3H,s,OCH_3); 3.39(1H,d(17.5Hz),$\underline{H}$CH); 3.13 (3H,s,NCH_3); 3.08(1H,d(8Hz),$\underline{H}$CH); 2.95(1H,d(8Hz),$\underline{H}$CH); 2.79(3H,s,NCH_3); 2.34(1H,d(17.5Hz),$\underline{H}$CH) ppm.

^{13}C nmr ($CDCl_3$): 167.2, 162.9, 159.7, 131.1(2), 125.6, 114.2(2), 85.4, 55.3, 51.4, 44.7, 33.3, 27.3 ppm.

hrms: m/e calcd. for $C_{14}H_{18}N_2O_4$: 278.1267; found: 278.1258(1%); 261($C_{14}H_{17}N_2O_3$)11; 260($C_{14}H_{16}N_2O_3$)8; 157($C_6H_9N_2O_3$)23; 121(C_8H_9O)100.

Methyl-6-(1,3-dihydro-4,6-dimethoxy-7-methyl-3-oxo-5-isobenzylfuranyl) -4-methyl-4-hexenoate, (14a)

Compound 14 (10 mg) was dissolved in dichloromethane (1.5 mL) and 0.3 M diazomethane was added dropwise with stirring at room temperature until the yellow colour persisted. The solvents were removed to give compound 14a in quantitative yield.

Formula (MW): $C_{19}H_{24}O_6$ (348).

ir ($CHCl_3$ cast): 2920, 2860, 1764, 1742, 1130 cm^{-1}.

^1H nmr ($CDCl_3$): 5.15(1H,d(7Hz),=CH); 5.11(2H,s,OCH_2); 4.03(3H,s,$ArOCH_3$); 3.75(3H,s,$ArOCH_3$); 3.59(3H,s,OCH_3); 3.39(2H,d(7Hz),CH_2); 2.34(4H,m, CH_2CH_2); 2.17(3H,s,$ArCH_3$); 1.80(3H,s,=CCH_3) ppm.

^{13}C nmr ($CDCl_3$): 174.3, 169.6, 163.5, 147.6, 134.5, 129.6, 124.5, 123.6, 120.8, 113.2, 69.5, 63.3, 61.6, 52.0, 35.4, 33.5, 24.2, 16.8, 12.1 ppm.

hrms: m/e calcd. for $C_{19}H_{24}O_6$: 348.1573; found: 348.1577 (69%); 316($C_{18}H_{20}O_5$)29; 301($C_{17}H_{17}O_5$)12; 275($C_{16}H_{19}O_4$)30; 273 ($C_{16}H_{17}O_4$)22; 261($C_{15}H_{17}O_4$)26; 243($C_{15}H_{15}O_3$)49; 221($C_{12}H_{13}O_4$)100; 209($C_{11}H_{13}O_4$)19; 207($C_{11}H_{11}O_4$)45; 159($C_{10}H_7O_2$)18; 91(C_7H_7)21.

cims: 366(M + NH_4^+).

N,N-dimethyl-6-methylthio-3-(4'-methoxybenzylidene)-piperazine-2,5-dione (29)

Compound 20 (ca. 3 mg) was heated under reflux in acetone (5 mL) in the presence of methyl iodide (excess) and Na_2CO_3 (ca. 2 mg) for 4 days. The reaction mixture was acidified and extracted with chloroform. The chloroform extract was concentrated to give compound 29 (1 mg).

Formula (MW): $C_{15}H_{18}N_2O_3S$ (306)

ir (cast); 2920, 1710, 1680, 1620, 1602, 1510, 1460, 1422, 1370, 1253, 1175, 1025, 825, 750 cm^{-1}.

uv (MeOH) λmax 214(16,300), 228(13,900), 277(4,284), 300(shl) nm.

^1H nmr ($CDCl_3$): 7.25(2H,d(8Hz),ArH); 7.16(1H,s,=CH); 6.90(2H,d(8Hz), ArH); 4.76(1H,s,CH); 3.83(3H,s,OCH_3); 3.14(3H,s,NCH_3); 2.94(3H,s,NCH_3), 2.29(3H,s,SCH_3) ppm.

hrms: m/e calcd. for $C_{15}H_{18}N_2O_3S$: 306.1038; found: 306.1042 (56%); 259($C_{14}H_{15}N_2O_3$)100; 231($C_{13}H_{15}N_2O_2$)86; 146(C_9H_8O)44; 121(C_8H_9O)21.

Isolation of Compound 36

Compound 36 was obtained from the ethyl acetate extract of the broth of still cultures of P. brevi-compactum. Flash chromatography of the crude (0.5 g) over silica gel (gradient elution, methanol) gave a polar fraction which contained pure, crystalline 36 (5 mg).

tlc (compound 36): R_f 0.15 (chloroform:methanol:acetic acid; 189:10:1). It is best visualised with $NaIO_4/KMnO_4$.

6-Hydroxy-3-methylthio-3-(4'-(3"-methyl-2"-butenoxy) phenylmethyl) piperazine-2,5-dione (36)

Formula (MW): $C_{17}H_{22}N_2O_4S$ (350).

MP: >290°C, decomposes (chloroform; fine white crystals).

ir ($CHCl_3$ cast): 3325, 3200, 3090, 3055, 2975, 2880, 1679 (strong), 1510, 1450, 1060, 820 cm^{-1}.

^1H nmr (DMSO-$\underline{d}_6$): 8.82(1H,s,NH); 8.61(1H,d(4Hz),NH; 7.14(2H,d(9Hz), ArH); 6.79(1H,d(6Hz),OH); 6.76(2H,d(9Hz),ArH); 5.39(1H,bt(8Hz),=CH); 4.44(2H,d(8Hz),OCH_3); 4.44(1H,dd(6,4Hz),CH); 3.33(1H,d(14Hz),$\underline{H}CH$); 2.86(1H,d(14Hz),$\underline{H}CH$); 2.18(3H,s,SCH_3); 1.73(3H,s,=CCH_3); 1.68(3H,s, =CCH_3) ppm.

The signals at 8.82, 8.61 and 6.79 ppm are exchangeable in D_2O.

hrms: m/e calcd. for $C_{17}H_{22}N_2O_4S$: 350.1301; found: 350.1300 (1%); 282 ($C_{12}H_{14}N_2O_4S$)9; 264($C_{12}H_{12}N_2O_3S$)7; 235($C_{11}H_{11}N_2O_4$)19; 234($C_{11}H_{10}N_2O_4$)17; 175($C_{12}H_{15}O$)14; 158($C_5H_6N_2O_2S$)4; 107(C_7H_7O)100; 69(C_5H_9)40.

cims: 368($M + NH_4^+$), 386($M + N_2H_8^+$).

Isolation of Compounds 43 and 47

Crude ethyl acetate mycelium extract (*ca*. 2g) from still cultures of *P. brevi-compactum* was divided into three fractions by flash chromatography (gradient elution, petroleum ether-dichloromethane-ethyl acetate-methanol). The third fraction (*ca*. 400 mg, $R_f \leq 0.3$, chloroform:methanol:acetic acid; 94:5:1) was chromatographed over silica gel (40g, $CHCl_3$). Compound 43 (purified by ptlc, chloroform:methanol:acetic acid, 89:10:1) (3 mg) was obtained from the second fraction and compound 47 (5 mg) was obtained as crystals from the final elution of the column with methanol.

tlc (compound 43): R_f 0.5 (chloroform:methanol:acetic acid:, 89:10:1) uv visible.

tlc (compound 47): R_f 0.3 (chloroform:methanol:acetic acid; 89:10:1) red-blue spot.

(S)N-(1-hydroxymethyl-2-phenylethyl) benzamide (43)

Formula (MW): $C_{16}H_{17}NO_2$ (255).

Mp: 172-173°C (dichloromethane, clear crystals).

$[\alpha]_D^{26}$: -73° (*c* 0.33, pyridine).

ir ($CHCl_3$ cast): 3340, 2920, 2850, 1637, 1536, 1452, 1290, 1030, 743, 700 cm^{-1}.

uv (MeOH) λmax (ε): 236(30,000)nm.

^{1}H nmr ($CDCl_3$/DMSO-$\underline{d}_6$): 7.82(2H,m, ArH); 7.74(1H,bd(8Hz),NH); 7.40(3H,m,ArH); 7.24(5H,m,ArH); 4.54(1H,t(5.5Hz),OH); 4.26(1H m(W1/2 = 17Hz),CH); 3.58(2H,dd(4.5,5.5Hz),OCH_2); 2.94(2H,d(7Hz), $ArCH_2$)ppm.

^{13}C nmr (DMSO-$\underline{d}_6$): 166.1, C=O; 139.4, 134.8, ArC; 131.0, 129.1(2), 128.1(4), 127.2(2), 125.9, ArCH; 62.9, CH_2; 53.2, CH; 36.5, CH_2 ppm.

hrms: m/e calcd. for $C_{16}H_{17}NO_2$: 255.1260, found: 255.1261 (3%); 224($C_{15}H_{14}NO$)4: 164($C_9H_{10}NO_2$)29; 151($C_8H_9NO_2$)23; 107(C_7H_7O)47; 105(C_7H_5O)94; 77(C_6H_5)36; 69(C_5H_9)26; 59(C_3H_7O)100.

cims: 256(M + H^+), 273(M + NH_4^+)

Cerevisterol (47; 3β,5α,6β,22E-ergosta-7,22-dione-3,5,6-triol)

Formula (MW): $C_{28}H_{46}O_3$ (430).

Mp: 257-259°C (methanol, white crystals).

$[\alpha]_D^{26}$: -75° ($\underline{c}$ 0.62, pyridine).

ir ($CHCl_3$ cast): 3610, 3450, 3310, 2980, 2860, 1659, 1459, 1448 1383, 1370, 1028, 968, 939, 865 cm^{-1}.

^{1}H nmr (DMSO-$\underline{d}_6$): 5.22(2H,m,(W1/2 = 11Hz)=CH); 5.09(1H,d(6Hz), =CH); 4.50(1H,d(5Hz),OH); 4.23(1H,d(6Hz),OH); 3.78(1H,bs(W1/2 = 26Hz),OCH); 3.60(1H,s,OH); 3.34(1H,m,CH); 0.99(3H,d(7Hz),CH_3); 0.91(3H,s, CH_3); 0.88(3H,d(7Hz),CH_3); 0.81(3H,d(6Hz),CH_3); 0.80(3H,d(6Hz)CH_3); 0.55 (3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{28}H_{44}O_2$(M^+-H_2O):412.3341; found: 412.3332 (26%); 394($C_{28}H_{42}O$)20; 383($C_{27}H_{43}O$)18; 379($C_{27}H_{39}O$)30; 376($C_{28}H_{40}$)5; 269($C_{19}H_{25}O$)17; 251($C_{19}H_{23}$)25; 197($C_{15}H_{17}$)9; 175($C_{12}H_{15}O$)10; 95(C_7H_{11})25; 93(C_7H_9)27; 91(C_7H_7)24; 83(C_6H_{11})22; 81(C_6H_9)42;

79(C_6H_7)23; 69(C_5H_9)100, 67(C_5H_7)29; 55(C_4H_7)79.
cims: 448($M + NH_4^+$).

3,6-Diacetoxy-3β,5α,6β,22E ergosta-7,2,2-diene-5-ol (48)

Compound 47 (5 mg) was added to dichloromethane (1.5 mL), pyridine (0.25 mL) and acetic anhydride (0.25 mL) and stirred overnight. The reaction was quantitative by tlc and provided 3.6 mg after concentration and recrystallisation from dichloromethane.

Formula (MW): $C_{32}H_{50}O_5$ (514).
Mp: 165-166°C (MeOH, white crystals).
$[\alpha]_D^{26}$: -150° (c 0.36, pyridine).
ir ($CHCl_3$ cast): 3450, 2955, 2865, 1734, 1713, 1459, 1369, 1240, 1025, 960, 940, 803, 708 cm^{-1}.
^1H nmr ($CDCl_3$): 5.20(4H,m); 4.82(1H,m); 2.04(3H,s,$COCH_3$); 2.01 (3H,s,$COCH_3$); 1.06(3H,s,CH_3); 0.94(3H,s,CH_3); 0.89(3H,s,CH_3); 0.85(3H,d(3Hz),3H); 0.79(3H,d(3Hz),CH_3); 0.58(3H,s,CH_3) ppm.
hrms: m/e calcd. for $C_{30}H_{46}O_3$ (M^+-CH_3COOH): 454.3447; found: 454.3438 (5%); 436($C_{30}H_{44}O_2$)39; 394($C_{28}H_{42}O$)55; 379($C_{27}H_{39}O$)11; 376($C_{28}H_{40}$)100; 251($C_{19}H_{23}$)38; 81(C_6H_9)18; 69(C_5H_9)52; 55(C_4H_7)29.
cims: 532($M + NH_4^+$).
tlc: R_f 0.9 (chloroform:methanol:acetic acid; 89:10:1) red-blue spot.

Isolation of p-hydroxybenzaldehyde (49)

The first fermentation culture of P. brevi-compactum was extracted with ethyl acetate. The crude organic mixture (ca. 5 g) was dissolved in ether (300 mL) and partitioned into its acidic, basic and neutral compounds. (2 x 150 mL 1N HCl, 2 x 150 mL sat. $NaHCO_3$, 2 x 150 mL 5% NaOH. After neutralisation the aqueous portions were re-extracted into chloroform and all organic solutions washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The weak acids fraction provided compound 49 (4 mg) after column chromatography (chloroform).

Formula (MW): $C_7H_6O_2$ (122).

Mp: 111-113°C (benzene/petroleum-ether, white crystals).

ir ($CHCl_3$ cast): 3580, 3020, 2925, 2855, 2355, 1687, 1602, 1587, 1159 cm^{-1}.

^1H nmr ($CDCl_3$): 9.89(1H,s,CHO); 7.87(2H,d(9Hz),ArH); 6.99(2H,d(9Hz), ArH); 6.00(1H,bs(W1/2 = 16Hz),OH) ppm.

hrms: m/e calcd. for $C_7H_6O_2$: 122.0368; found: 122.0366 (94%); 121($C_7H_5O_2$)100; 93(C_6H_5O)50; 65(C_5H_5)37.

tlc: 0.5(chloroform:methanol:acetic acid; 89:10:1) purplish spot after vigourous heating.

Isolation of Compounds 50, 57 and 62

The compounds were only isolated from the ethyl acetate extract of our first fermentation growth of P. brevi-compactum. The crude extract from 3 L of fermentor culture was separated over silica gel (flash chromatography) by gradient elution (petroleum ether-dichloromethane-ethyl acetate-methanol) to provide mostly compound 62 in fraction 6 (40% ethyl acetate in dichloromethane) and compounds 50 and 57 in the seventh fraction (60% ethyl acetate in dichloromethane).

Compounds 50 and 57 were purified by ptlc (chloroform:methanol:acetic acid; 89:10:1) providing 3 mg of 50 and 2 mg of 57. Compound 62 was purified by fractional crystallisation (ether/petroleum-ether) which separated out a white crystalline impurity leaving 62 (ca. 60 mg) in solution.

tlc (compound 50): R_f 0.15 (chloroform:methanol:acetic acid; 89:10:1), bright blue spot under uv light.

tlc (compound 57): R_f 0.4 (chloroform:methanol:acetic acid; 89:10:1), uv visible.

tlc (compound 62): R_f 0.45 (chloroform:methanol:acetic acid; 89:10:1), dark brown spot.

2(1-Oxo-2-hydroxypropyl) aminobenzamide (50)

Formula (MW): $C_{10}H_{12}N_2O_3$ (208).

Mp: 140-142°C (benzene/methanol, white crystals).

ir (MeOH cast): 3300, 2890, 2860, 1680, 1620, 1590, 1540, 1450, 1400, 1300, 1120, 760, 630 cm^{-1}.

uv (Ethanol) λmax (ε): 216.5(31,700); 252(19,900), 260(shoulder); 299(4690) nm.

^{1}H nmr (DMSO-$\underline{d}_6$): 12.00(1H,s,NH); 8.60(1H,dd(8.5,1Hz),ArH); 8.15(1H, bs(W1/2 = 10Hz),NH); 7.77(1H,dd(8,2Hz),ArH); 7.56(1H,bs,NH); 7.50(1H, td(8,2Hz),ArH; 7.12(1H,dt(8,2Hz),ArH); 5.92(1H,bs(W1/2 = 22Hz),OH); 4.11(1H,q(7Hz),OCH); 1.30(3H,d(7Hz),CH_3) ppm.

^{13}C nmr (MeOH-$\underline{d}_4$): 176.6, 173.4, 139.6, 133.3, 129.5, 124.5, 122.7, 122.2, 69.8, 21.1 ppm.

hrms: m/e calcd. for $C_{10}H_{12}N_2O_3$: 208.0848; found: 208.0847 (21%); 163($C_8H_7N_2O_2$)91; 146($C_8H_4NO_2$)100; 136($C_7H_8N_2O$)30; 119(C_7H_5NO)55; 90(C_6H_4N)29; 65(C_5H_5)18.

2-(1,2-Dioxopropyl) aminobenzamide (57)

Formula (MW): $C_{10}H_{10}N_2O_3$ (206).

Mp: 198-200°C (dichloromethane, white crystals).

ir (MeOH cast): 3400, 3300, 1725, 1685, 1655, 1605, 1590, 1520, 1385, 1360, 755, 625, 605, 540 cm^{-1}.

uv (MeOH) λmax(ε): 218(9040), 247.5(6980), 260(shoulder), 303(3780) nm.

^{1}H nmr (MeOH-d_4) (methanol forms a hemi-ketal with compound 57 therefore the spectrum is divided into two parts: a) for the α-diketone form (57) and b) for the hemi-ketal (60):

a) 8.61(1H,dd(8,2Hz),ArH); 7.78(1H,dd(8,2Hz),ArH); 7.53(1H,td(8,2Hz), ArH); 7.18(1H,td(8,2Hz),ArH); 2.47(3H,s,$COCH_3$) ppm.

b) 8.55(1H,dd(8,2Hz),ArH); 7.74(1H,dd(8,2Hz),ArH); 7.50(1H,td(8,2Hz), ArH); 7.15(1H,td(8,2Hz),ArH); 1.56(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{10}H_{10}N_2O_3$: 206.0691; found: 206.0691 (2%); 163($C_8H_7N_2O_2$)80; 146($C_8H_4NO_2$)100; 119(C_7H_5NO)10; 90(C_6H_4N)27.

The C_{22} Compound 62 (11-(1'-epoxy-5'-hydroxy-4'-hydroxymethylcyclo-3'-hexen-2'-one)-$\Delta^{8(12)}$-drimene)

Formula (MW): $C_{22}H_{32}O_4$ (360).

Mp: 124-126°C (ether/petroleum-ether, white crystals).

$[\alpha]_D^{26}$: +35° (c 0.22, $CHCl_3$).

ord (c 0.43, MeOH), 26°C: $[\phi]_{530}$ 0°, $[\phi]_{360}$ + 4650°(peak), $[\phi]_{340}$ 0° (intersects), $[\phi]_{300}$ -9040°(shoulder), $[\phi]_{250}$ - 13,800°(trough), $[\phi]_{230}$ 0° (intersects).

cd(c 0.43, MeOH), 26°C: $[\theta]_{381}$ 0 ; $[\theta]_{332}$ + 9,540; $[\theta]_{290}$ (inflection); $[\theta]_{226}$ 0 ; $[\theta]_{253}$ - 5,520; $[\theta]_{220}$ 0.

ir ($CHCl_3$ cast): 3400, 3080, 2940, 1680, 1460, 1440, 1385, 1275, 1200, 1100, 1030, 882, 665 cm^{-1}.

uv (meOH) λmax(ε): 221(4,650), 239(8,250) nm.

^{1}H nmr ($CDCl_3$): 5.96(1H,bs(W1/2 = 3Hz),=CH); 4.81(1H,bs(W1/2 = 3Hz), =C$\underline{H}$H); 4.57(2H,bs,=C$\underline{H}$H,OC$\underline{H}$); 4.31(2H,bs(W1/2 = 3Hz),OCH_2); 3.71(1H,d (3Hz),OCH); 0.89(3H,s,CH_3); 0.82(3H,s,CH_3); 0.72(3H,s,CH_3) ppm.

^{13}C nmr ($CDCl_3$): 194.3, C+O; 158.9, 149.4, =C; 120.2, =CH; 106.8, $=CH_2$; 65.3, OCH; 61.8, OCH_2; 61.2, OCH; 60.7, O-C; 55.7, 51.8, CH; 42.3, CH_2; 39.9, C; 39.0, 38.3, CH_2; 33.7, C; 33.6, CH_3; 24.7, CH_2; 21.8, CH_3; 21.3, 19.5, CH_2; 14.7, CH_3 ppm.

hrms: m/e calcd. for $C_{22}H_{32}O_4$: 360.2301; found: 360.2290 (2%); 345($C_{21}H_{29}O_4$)2; 342($C_{22}H_{30}O_3$)10; 327($C_{21}H_{27}O_3$)7; 313($C_{21}H_{29}O_2$)6; 206($C_{12}H_{14}O_3$)3; 205($C_{15}H_{25}$)5; 191($C_{14}H_{23}$)12; 177($C_{13}H_{21}$)12; 167($C_8H_7O_4$)15; 152($C_8H_8O_3$)19; 137($C_{10}H_{17}$)86; 123(C_9H_{15})32; 109(C_8H_{13})28; 107(C_8H_{11})25; 105(C_8H_9)24; 95(C_7H_{11})49; 93(C_7H_9)29; 91(C_7H_7)33; 81(C_6H_9)60; 79(C_6H_7)33; 77(C_6H_5)25; 69(C_5H_9)71; 67(C_5H_7)38; 66(C_4H_7)70.

cims: 378($M + NH_4^+$).

2(1-Oxo-2-acetoxypropyl) aminobenzamide (55)

Ca. 3 mg of compound 50 was stirred with 10 drops of acetic anhydride and 20 drops of pyridine in chloroform for 3 hours. The reaction mixture was concentrated and placed under high vacuum (0.1 torr, room temperature, 24 hours) which provided compound 55 quantitatively.

Formula (MW): $C_{12}H_{14}N_2O_4$ (250).

Mp: 126-128°C (chloroform, white crystals).

ir ($CHCl_3$ cast): 3350, 3220, 2995, 2930, 1740, 1670, 1620, 1585, 1530, 1450, 1390, 1230, 755 cm^{-1}.

^1H nmr ($CDCl_3$): 11.78(1H,bs(W1/2 = 7Hz), NH); 8.64(1H,d(8Hz),ArH); 7.54(1H,dd,ArH); 7.50(1H,dt,ArH); 7.08(1H,dt(8,1.5Hz),ArH); 6.20(2H, bs(W1/2 = 27Hz),NH); 5.30(1H,q(7Hz),OCH); 2.25(3H,s,$COCH_3$); 1.54(3H, d(7Hz),CH_3) ppm.

hrms: m/e calcd. for $C_{12}H_{14}N_2O_4$: 250.0954; found: 250:0959 (40%); 163($C_8H_7N_2O_2$)94; 146($C_8H_4NO_2$)100; 136($C_7H_8N_2O$)50; 120(C_7H_6NO)20;

119(C_7H_5NO)59; 90(C_6H_4N)37; 87($C_4H_7O_2$)25; 65(C_5H_5)29.

2-(1-Hydroxyethyl)-4-quinazalinone (56)

Compound 55 (ca. 3.5 mg) was stirred in 2N NaOH (1.5 mL) for 10 minutes, acidified (HCl) and extracted with ethylacetate. The ethyl acetate extract was dried and concentrated to give compound 56 (2 mg).

Formula (MW): $C_{10}H_{10}N_2O_2$ (190).

ir ($CHCl_3$ cast): 3100(broad), 1686(s), 1625(m), 1609(m), 1465(m), 775 cm^{-1}.

ir ($CHCl_3$): 3620, 3364, 30.0, 2970, 1677(s), 1624(w), 1610(w), 1040 cm^{-1}

uv (MeOH) λmax (ε): 225(24,000), 232(shoulder), 264(8,000), 272(shoulder), 303(4,600), 314(3,900) nm.

^1H nmr (DMSO-d_6): 8.51(1H,dd(8,2Hz),ArHO; 7.90-7.40(4H,m); 4.59(1H,q(6Hz),OCH); 2.80(1H,b,OH); 1.43(3H,d(6Hz),CH_3) ppm.

hrms: m/e calcd. for $C_{10}H_{10}N_2O_2$: 190.0742; found: 190.0739 (100%); 175($C_9H_7N_2O_2$)51; 173($C_{10}H_9N_2O$)73; 147($C_8H_7N_2O$)69; 146($C_8H_6N_2O$)21; 145($C_8H_5N_2O$)20; 119(C_7H_5NO)48; 90(C_6H_4N)48.

tlc: 0.45 (chloroform:methanol:acetic acid; 89:10:1), uv visible.

11-(1'-epoxy-5'-acetoxy-4'-acetoxymethylcyclo-3'-hexen-2'-one) $\Delta^{8(12)}$-drimene (63)

Compound 62 24 mg) was dissolved in dichloromethane (2 mL) and treated with pyridine (5 drops) and acetic anhydride (0.5 mL) with stirring at room temperature. The reaction which was complete after 10 minutes was evapourated and dried under high vacuum. Pure diacetyl compound (63) was obtained after ptlc (16.4 mg) over silica gel (chloroform:methanol:acetic acid; 94:5:1)

Formula (MW): $C_{26}H_{36}O_6$ (444).

ir ($CHCl_3$ cast): 2960, 1750, 1685, 1645, 1460, 1440, 1370, 1240, 1100, 1040, 885, 760 cm^{-1}.

uv (MeOH) λmax (ϵ): 229(10,400) nm.

^1H nmr ($CDCl_3$): 6.04(1H,m(W1/2 = 4Hz),=CH); 5.84(1H,bs(W1/2 = 6Hz), OCH); 4.82(1H,bs(W1/2 = 4Hz),=CHH); 4.77(1H,bd(15Hz),OCHH); 4.56(1H, bd(15Hz),OCHH); 4.48(1H,bs(W1/2 = 4Hz),=CHH); 3.75(1H,d(3Hz),OCH); 2.19(3H,s,$COCH_3$); 2.08(3H,s,$COCH_3$); 0.87(3H,s,CH_3); 0.80(3H,s,CH_3); 0.71(3H,s,CH_3) ppm.

^{13}C nmr ($CDCl_3$): 192.1, 169.9, 169.7, 148.7, 147.3, 124.0, 106.6, 66.3, 62.1, 59.7, 56.9, 55.2, 51.0, 41.8, 39.4, 38.7, 37.8, 33.3(2), 24.1, 21.5, 20.5, 20.3, 20.0, 19.1, 14.3 ppm.

hrms: m/e calcd. for $C_{26}H_{36}O_6$: 444.2512; found: 444.2511 (1%); 429($C_{25}H_{33}O_6$)2; 384($C_{24}H_{34}O_4$)4; 342($C_{22}H_{30}O_3$)14; 324($C_{22}H_{28}O_2$)20; 309($C_{21}H_{25}O_2$)11; 295($C_{21}H_{27}O$)5; 233($C_{13}H_{13}O_4$)6; 205($C_{12}H_{13}O_3$)9; 203($C_{15}H_{23}$)11; 195($C_{10}H_{11}O_4$)16; 191($C_{14}H_{23}$)8; 189($C_{14}H_{21}$)24; 137($C_{10}H_{17}$)100; 136($C_{10}H_{16}$)22; 123(C_9H_{15})37; 121(C_9H_{13})30; 119(C_9H_{11})23; 109(C_8H_{13})30; 107(C_8H_{11})29; 105(C_8H_9)25; 95(C_7H_{11})59; 93(C_7H_9)32; 91(C_7H_7)30; 81(C_6H_9)66; 79(C_6H_7)31, 69(C_5H_9)69; 67(C_5H_7)32; 55(C_4H_7)51.

cims: 462(M + NH_4^+).

tlc: R_f 0.9 (chloroform:methanol:acetic acid; 89:10:1), brown spot.

11-(1'-epoxy-4'-hydroxymethylcyclo-3'-hexen-2',5'-dione) -$\Delta^{8(12)}$-drimene (74)

CrO_3 (33.5 mg) was added to a cooled (5°C) solution of dry pyridine (53 mg) in dichloromethane (10 mL) and allowed to stir for 24 hours as it warmed to room temperature. The red solution was cooled to 5°C and compound 62 (10 mg) dissolved in dichloromethane was added rapidly. The stirred solution was allowed to warm to room temperature, diluted with dichloromethane, filtered through celite, and CH_2Cl_2 removed in vacuo. Ptlc of the residue by double elution with 30% petroleum ether in ether provided compound 74 (3 mg) separating it from its isomer, the aldehyde compound 75.

Formula (MW): $C_{22}H_{30}O_4$ (358).

ir ($CHCl_3$ cast): 3440, 2960, 1710(shoulder); 1687, 1460, 1440, 1385, 1200, 885 cm^{-1}.

^1H nmr ($CDCl_3$): 6.65(1H,t(2Hz),=CH); 4.84(1H,d(1Hz),=C$\underline{H}$H); 4.56(1H, dd(16,2Hz),OC$\underline{H}$H); 4.53(1H,d(1Hz),=C$\underline{H}$H); 4.36(1H,dd(16,2Hz); 3.75(1H, s,OCH); 0.87(3H,s,CH_3); 0.80(3H,s,CH_3); 0.71(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{22}H_{30}O_4$: 358.2144; found: 358.2146 (4%); 343($C_{21}H_{27}O_4$)8; 340($C_{22}H_{28}O_3$)5; 273($C_{16}H_{17}O_4$)4; 204($C_{15}H_{24}$)7; 203($C_{15}H_{23}$)17; 189($C_{14}H_{21}$)20; 137($C_{10}H_{17}$)100; 123(C_9H_{15})31; 109(C_8H_{13})22; 95(C_7H_{11})37; 91(C_7H_7)20; 81(C_6H_9)41; 69(C_5H_9)48; 67(C_5H_7)21; 55(C_4H_7)35.

tlc: R_f 0.4 (chloroform:methanol:acetic acid; 89:10:1), brown spot.

11-(1'-epoxy-4'-acetoxymethylcyclo-3'-hexen-2',5'-dione) -$\Delta^{8(12)}$-drimene (74a)

Compound 74 (2 mg) was dissolved in CH_2Cl_2 (1.5 mL) and stirred with acetic anhydride (10 drops) and pyridine (20 drops) at room temperature for 30 minutes. The solvents were evapourated in vacuo providing compound 74 acetate (2 mg).

Formula (MW): $C_{24}H_{32}O_5$ (400).

ir ($CHCl_3$ cast); 2920, 1750, 1685, 1640, 1460, 1440, 1365, 1220, 880 cm^{-1}.

^1H nmr ($CDCl_3$); 6.51(1H,t(2Hz),=CH); 4.96(1H,dd(16,2Hz),OC$\underline{H}$H); 4.82(1H,bs,=C$\underline{H}$H); 4.77(1H,dd(16,2Hz),OC$\underline{H}$H); 4.52(1H,bs,=C$\underline{H}$H); 3.76(1H, s,OCH); 2.13(3H,s,COCH$_3$); 0.88(3H,s,CH$_3$); 0.81(3H,s,CH$_3$); 0.71(3H, s,CH$_3$) ppm.

hrms: m/e calcd. for $C_{24}H_{32}O_5$: 400.2250; found: 400.2259 (2%); 385($C_{23}H_{29}O_5$)6; 372($C_{23}H_{32}O_4$)4; 322($C_{22}H_{26}O_2$)10; 233($C_{16}H_{25}O$)3; 204($C_{15}H_{24}$)4; 189($C_{14}H_{21}$)14; 137($C_{10}H_{17}$)62; 123(C_9H_{15})31; 109(C_8H_{13})29; 107(C_8H_{11})20; 105(C_8H_9)21; 97(C_7H_{13})30; 95(C_7H_{11})45; 93(C_7H_9)23; 91(C_7H_7)28; 85(C_6H_{13})35; 83(C_6H_{11})37; 81(C_6H_9)51; 79(C_6H_7)27; 71(C_5H_{11})52; 69(C_5H_9)100; 67(C_5H_7)33; 57(C_4H_9)89; 55(C_4H_7)83.

11-(1'-epoxy-5'-hydroxy-4'-oxomethylcyclo-3'-hexen-2'-one) -$\Delta^{8(12)}$-drimene (75)

Compound 62 (10 mg) was stirred in anhydrous ether (15 mL) with activated MnO_2 (60 mg) for 2.5 hours at room temperature. The reaction mixture was filtered through celite and the filtrate evapourated *in vacuo*. The residue provided compound 74 (3 mg) and 75 (3 mg) after separation by ptlc (double elution with 30% petroleum-ether in ether over silica gel).

Formula (MW): $C_{22}H_{30}O_4$ (358).

ir ($CHCl_3$ cast); 3420, 2920, 1685, 1455, 1440, 1380, 1090, 1035, 880, 755 cm^{-1}.

^{1}H nmr ($CDCl_3$): 9.72(1H,s,CHO); 6.48(1H,d(1Hz),=CH); 4.98(1H,m(W1/2 = 8Hz),OCH); 4.84(1H,bs,=C$\underline{H}$H); 4.55(1H,bs,=CH$\underline{H}$); 3.61(1H,d(4Hz),OCH); 0.88(3H,s,CH$_3$); 0.81(3H,s,CH$_3$); 0.72(3H,s,CH$_3$) ppm.

hrms: m/e calcd. for $C_{22}H_{30}O_4$: 358.2144; found: 358.2155 (14%); 343($C_{21}H_{27}O_4$)22; 340($C_{22}H_{28}O_3$)11; 311($C_{21}H_{27}O_2$)14; 273($C_{16}H_{17}O_4$)10; 204($C_{15}H_{24}$)6: 203($C_{15}H_{23}$)11; 191($C_{14}H_{23}$)19; 189($C_{14}H_{21}$)24; 137($C_{10}H_{17}$)100; 123(C_9H_{15})40; 121(C_9H_{13})23; 119(C_9H_{11})23; 109(C_8H_{13})29; 107(C_8H_{11})25;

105(C_8H_9)36; 95(C_7H_{11})49; 93(C_7H_9)33; 91(C_7H_7)55; 81(C_6H_9)61; 79(C_6H_7)44; 77(C_6H_5)35; 69(C_5H_9)87; 67(C_5H_7)43; 55(C_4H_7)76; 55(C_3H_3O)24; 53(C_4H_5)29

tlc: R_f 0.4 (chloroform;methanol:acetic acid; 89:10:1), brown spot

11(1'-epoxy-2',5'-dihydroxy-4'-hydroxymethylcyclo-3'-hexene) -$\Delta^{8(12)}$-drimene (76)

A solution of compound 62 (8.5 mg) in anhydrous ether (10 mL) was cooled to 0°C. Two spatula tips of lithium aluminium hydride were added and the reaction mixture was allowed to stir over 1 hour as the reaction warmed to room temperature. Water (10 mL) was added and the mixture was extracted with ether (3 x 10 mL). The combined ether extracts were washed with sat. NaCl and dried over anhydrous $MgSO_4$. Filtration and concentration followed by separation on an analytical tlc plate (silica gel; chloroform:methanol:acetic acid; 84:15:1) provided compound 76 (3 mg).

Formula (MW): $C_{22}H_{34}O_4$ (362).

ir ($CHCl_3$ cast): 3380, 2920, 1640, 1455, 1435, 1380, 1360, 1205, 1020, 875, 750 cm^{-1}.

^1H nmr ($CDCl_3$): 5.51(1H,m,=CH); 4.84(1H,m,=CHH); 4.81(1H,M,OCH); 4.64(1H,bs,=CHH); 4.43(1H,m,OCH); 4.22(*ca*. 4H,bs(W1/2 = 7Hz),OCH_2,OH); 3.53(1H,d(3.5Hz),OCH); 0.87(3H,s,CH_3); 0.80(3H,s,CH_3); 0.70(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{22}H_{32}O_3(M^+ - H_2O)$: 344.2351; found: 344.2353 (4%); 326($C_{22}H_{30}O_2$)6; 206($C_{15}H_{26}$)5%; 204($C_{15}H_{24}$)8; 191($C_{14}H_{23}$)42; 190($C_{14}H_{22}$)20; 189($C_{14}H_{21}$)22; 140($C_7H_8O_3$)15; 137($C_{10}H_{17}$)100; 136($C_{10}H_{16}$)24; 123(C_9H_{15})42; 123($C_7H_7O_2$)15; 121(C_9H_{13})31; 120(C_8H_8O)20; 109(C_8H_{13})37; 107(C_8H_{11})33; 95(C_7H_{11})60; 95(C_6H_7O)20; 93(C_7H_9)38; 91(C_7H_7)34; 81(C_6H_9)72;

79(C_6H_7)38; 69(C_5H_9)79; 67(C_5H_7)44; 55(C_4H_7)66.

11-(1'-epoxy-5'-hydroxy-4'-hydroxymethylcyclohexan-2'-one) drimane (77)

Compound 62 (10 mg) was dissolved in methanol (15 mL). This solution was shaken with 5% Pd-C (30 mg) on a Parr hydrogenator (H_2, 1 atm. at RT) for 5 hours. The reaction mixture was filtered through celite and concentrated *in vacuo*. The concentrate was separated by tlc (silica gel, chloroform:methanol:acetic acid; 89:10:1) providing pure compound 77 (3 mg).

Formula (MW): $C_{22}H_{36}O_4$ (364).

ir ($CHCl_3$ cast): 3400, 2920, 1730(shoulder), 1710, 1460, 1380, 1260, 1040 cm^{-1}.

^1H nmr ($CDCl_3$): 4.18(1H,bm,OCH); 3.72(2H,bm,OCH_2); 3.18(1H,d(3Hz),OCH); 0.92(3H,d(3Hz),CH_3); 0.83(6H,bs,CH_3); 0.81(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{22}H_{36}O_4$: 364.2593; found: 364.2603 (1%); 206($C_{15}H_{26}$)83; 191($C_{14}H_{23}$)46; 144($C_7H_{12}O_3$)40; 137($C_{10}H_{17}$)25; 131($C_6H_{11}O_3$)37; 126($C_7H_{10}O_2$)37; 123(C_9H_{15})100; 109(C_8H_{13})44; 99($C_5H_7O_2$)45; 97(C_7H_{13})20; 95(C_7H_{11})54; 83(C_6H_{11})25; 81(C_6H_9)68; 69(C_5H_9)85; 57(C_4H_9)27; 55(C_4H_7)82.

tlc: R_f 0.45 (chloroform:methanol:acetic acid; 89:10:1), brown spot.

Isolation of Compound 78

Compound 78 was isolated from the ethyl acetate extract of the broth of a 10 L fermentation culture of *G. abietina*. The crude extract (*ca*. 7 g) was separated over Sephadex LH-20 and a fraction (2.6 g, tubes 22 to 29) was chromatographed over silica gel (flash column, diameter 5 cm) by gradient elution (0 to 8% methanol in chloroform). Compound 78 was purified by two consecutive ptlc (1) double elution 1% MeOH/$CHCl_3$; 2) double elution 5% MeOH/$CHCl_3$) providing 4 mg of pure compound.

tlc (compound 78): R_f 0.7 (chloroform:methanol:acetic acid; 89:10:1), yellow spot.

The Acetone Adduct of Atrovenetinone (78; 3,5,7-trihydroxy-4,6-dioxo-1, 8,8,9-tetramethyl-5-(propan-2'-onyl)-5,6,8,9-tetrahydro-4H-phenaleno [1,2,b] furan

Compound 78 was shown to be an artifact produced during isolation and was also synthesised by stirring compound 85 (atrovenetinone, 20 mg) in acetone (2 mL) with 10 drops of acetic acid at room temperature for ca. 2 hours. The reaction was monitored by tlc until no compound 85 remained. The adduct 78 was purified by ptlc (silica gel; chloroform: methanol:acetic acid; 189:10:1) providing 13 mg.

Formula (MW): $C_{22}H_{22}O_7$ (398).

ir ($CHCl_3$ cast): 3400, 2960, 2930, 2850, 1730, 1710, 1635, 1605, 1455, 1380, 1335, 1310, 1290, 1200, 1170, 1140, 1060, 1030, 865, 745, 515 cm^{-1}

uv (MeOH) λmax (ε): 221(33,000); 230(27,000, shoulder); 252(30,000, shoulder); 260(41,000); 341(15,000); 364(11,000, shoulder) nm.

^1H nmr ($CDCl_3$): 13.32*(1H,s,OH); 12.80*(1H,s,OH); 6.76(1H,q(1Hz), ArH); 4.64*(1H,q(6.5Hz),OCH); 3.70(1H,bs,OH); 3.30*(2H,s,$COCH_2$); 2.78(3H,d(1Hz),$ArCH_3$); 2.23(3H,s,$COCH_3$); 1.52*(3H,s,CH_3); 1.46*(3H,d (6.5Hz),CH_3; 1.30*(3H,s,CH_3) ppm.

Compound 78 is a diastereomeric pair (C-2) and the downfield signals attributable to equivalent hydrogens of that pair are reported as indicated*.

^{13}C nmr ($CDCl_3$): 206.4*, 199.8, 197.6, C=O; 166.5, 166.3*, 165.7, 149.7, 137.8, 119.1*, ArC; 118.1, ArCH; 110.0, 105.8, 103.0, ArC; 92.1*, OCH; 77.7, OC; 52.2*, CH_2; 43.7, C; 31.1*, 25.9*, 24.3*, 20.7, 14.8, CH_3.

Compound 78 is a diastereomeric pair (C-2) and the downfield signals attributable to equivalent carbons of that pair are reported as indicated.

hrms: m/e calcd. for $C_{22}H_{22}O_7$: 398.1368; found: 398.1364 (45%); 383($C_{21}H_{19}O_7$)4; 355($C_{20}H_{19}O_6$)28; 313($C_{18}H_{17}O_5$)100; 297($C_{17}H_{13}O_5$)28; 157($C_{14}H_9O_5$)14.

Isolation of Compounds 79, 85, 97, 100, 101, and 105 to 110

In a typical separation, ethyl acetate extract of the broth of the fermentation culture of G. abietina (ca. 1 g) was chromatographed over Sephadex LH-20. The third fraction (tube #18 to 29) contained the bulk of the metabolites (260 mg), the fourth (tube #30 to 34) contained 33 mg of material similar to that in the third fraction but with more polar constituents and the fifth fraction (tube #35-58, 61 mg) was the last fraction to contain significant amounts of metabolites.

The third fraction was separated by flash chromatography (gradient elution: dichloromethane-ether-methanol) and gave the anhydride (79) (dichloromethane, 12 mg), the trione (85) (20% dichloromethane-ether, 60 mg). Upon further purification of an ether fraction (ptlc, chloroform: acetic acid; 95:5) pure compound 101 (3 mg) was obtained.

tlc (compound 79): R_f 0.9 (chloroform:methanol:acetic acid; 94:5:1) light spot, intense blue fluorescence under uv light.

tlc (compound 85): R_f 0.8 (chloroform:methanol:acetic acid; 189:10:1) green-yellow spot.

tlc (compound 101): R_f 0.45 (chloroform:petroleum-ether:acetic acid; 14:5:1 (double elution)), purple spot.

The fifth flash column fraction when separated by ptlc (chloroform: methanol:acetic acid, 89:10:1) provides ca. 3 mg mixtures containing compounds 105, 106, and 107; compounds 108 and 109; and compound 110.

tlc (compounds 105 to 107): R_f: 0.45 (chloroform:methanol; 85:15

(1 drop acetic acid)) light orange spot.

tlc (compounds 108 and 109): R_f 0.35 (chloroform:methanol; 85:15 (1 drop AcOH)), light orange spot.

tlc (compound 110): R_f 0.4 (chloroform:methanol; 85:15 (1 drop AcOH)) light orange spot.

On the other hand treatment of the fifth flash column fraction with diazomethane (0.3 M) in ether at room temperature gave a mixture from which 97 or 100 could be isolated. The addition of CH_2N_2 was monitored by tlc when the bright yellow fluorescing compound remained unchanged, an aliquot (uv light) was taken, the solvent was evapourated and the residue purified by ptlc (ether:petroleum-ether; 3:1 (1% acetic acid) and compound 97 (10 mg) was obtained.

The addition of CH_2N_2 was continued. Evapouration of the solvent led to isolation (2 mg) of a small quantity of the trimethyl ether (100) after ptlc (chloroform:methanol:acetic acid; 189:10:1)

tlc (compound 97): R_f 0.6 (ether:petroleum-ether; 3:1 (1 drop of acetic acid), uv visible.

tlc (compound 100): R_f 0.75 (ether:petroleum-ether; 3:1 (1 drop acetic acid) uv visible.

The Naphthalic Anhydride (79; 4,7-dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho[1,2-b]furan-5,6-dicarboxylic anhydride)

ir($CHCl_3$ cast); 2960, 1740, 1670, 1630, 1615, 1465, 1390, 1305, 1190, 1045, 875, 815, 765, 725, 665, 545 cm^{-1}.

^1H nmr ($CDCl_3$): 11.64(1H,s,OH); 11.42(1H,s,OH); 6.86(1H,s,ArH); 4.72(1H,q(6.5Hz),OCH); 2.82(1H,s,$ArCH_3$); 1.55(3H,s,CH_3); 1.50(3H,d (6.5Hz),CH_3); 1.32(3H,s,CH_3) ppm.

^{1}H nmr (pyridine-d_5): 6.95(1H,s,ArH); 4.60(1H,q(6Hz),OCH); 2.73(3H, s,ArCH$_3$); 1.55(3H,s,CH$_3$); 1.34(3H,d(6Hz),CH$_3$); 1.29(3H,s,CH$_3$) ppm

hrms: m/e calcd. for $C_{18}H_{16}O_6$: 328.0947; found: 328.0949, (34%); 313($C_{17}H_{13}O_6$)100; 295($C_{17}H_{11}O_5$)9; 285($C_{16}H_{13}O_5$)7; 269($C_{16}H_{13}O_4$)8.

Atrovenetinone (85; 3,7-dihydroxy-4,5,6-trioxo-1,8,8,9-tetramethyl-5,6,8,9-tetrahydro-4H-phenaleno [1,2-b] furan)

Formula (MW): $C_{19}H_{16}O_6$ (340).

Mp: 220-222°C (benzene/acetone, dark purple crystals).

ir (CH$_2$Cl$_2$ cast): 3390, 2955, 2920, 2860, 1720, 1634, 1605, 1458, 1382, 1140, 1055, 1025, 860, 825, 565, 535 cm^{-1}.

^{1}H nmr (DMSO-d_6): 14.09(1H,s,OH); 13.15(1H,s,OH); 6.89(1H,s,ArH); 4.79(1H,q(7Hz),OCH); 2.75(3H,s,ArCH$_3$); 1.50(3H,s,CH$_3$); 1.46(3H,d(7Hz), CH$_3$); 1.26(3H,s,CH$_3$) ppm.

^{1}H nmr (DMSO-d_6 + H$_2$O) (compound 85 hydrate, 91): 13.75(1H,s,OH); 12.95(1H,bs(W1/2 = 6Hz),OH); 7.58(2H,bs(W1/2 = 8Hz),OH); 6.84(1H,q (1Hz),ArH): 4.73(1H,q(7Hz),OCH); 2.73(3H,d(1Hz),ArCH$_3$); 1.48(3H,s, CH$_3$); 1.43(3H,d(7Hz),CH$_3$); 1.24(3H,s,CH$_3$) ppm.

^{13}C nmr (DMSO-d_6) (compound 85 hydrate, 91): 197.1, 196.1, 166.0*, 165.4*, 164.8*, 147.8, 136.7, 118.0, 117.5, 108.9, 104.8, 101.9, 91.3*, 87.9, 42.7, 25.1, 23.4*, 20.3, 14.2 ppm.

*These signals appear as pairs possibly as a result of tautomerisation; the downfield signals of each pair are recorded.

hrms: m/e calcd. for $C_{19}H_{16}O_6$: 340.0947; found: 340.0942 (28%); 327($C_{18}H_{15}O_6$)18; 312($C_{18}H_{16}O_5$)25; 297($C_{17}H_{13}O_5$)100; 269($C_{16}H_{13}O_4$)42.

cims: 368 (M + NH$_4^+$).

The 8-Hydroxynaphthalic Anhydride Methyl Ether (97; 4,8-dihydroxy-7-methoxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho [1,2-b] furan-5,6-dicarboxylic anhydride)

Formula (MW): $C_{19}H_{18}O_7$ (358).

Mp: 213-214°C (ether/petroleum-ether, yellow crystals).

$[\alpha]_D^{26}$ ($\underline{c}$ 0.11, chloroform): -16°.

ir ($CHCl_3$ cast): 3400, 2940, 1750, 1685, 1600, 1450, 1405, 1305, 1240, 1140, 1050, 800, 765 cm^{-1}.

uv (MeOH) λmax (ε): 211(28,400); 234(17,300); 257(26,900); 309(3,280); 356(8,360); 396(10,700, shoulder); 409(11,000) nm.

^{1}H nmr (CD_2Cl_2): 11.94(1H,s,OH); 6.47(1H,bs(W1/2 = 6Hz),OH); 4.73(1H, q(6.5Hz),OCH); 4.10(3H,s,OCH_3); 2.83(3H,s,$ArCH_3$); 1.56(3H,s,CH_3); 1.50(3H,d(6.5Hz),CH_3); 1.32(3H,s,CH_3) ppm.

^{1}H nmr (pyridine-d_5): 4.95(2H,bs (W1/2 = 16Hz),OH); 4.67(1H,q(6.5Hz), OCH); 4.12(3H,s,OCH_3); 3.09(3H,s,$ArCH_3$); 1.56(3H,s,CH_3); 1.45(3H,d (6.5Hz),CH_3); 1.34(3H,s,CH_3) ppm.

^{13}C nmr (pyridine-d_5): 166.7, 164.8, 163.1, 157.6, 156.1, 148.2, 131.9, 129.4, 120.5, 108.4, 107.1, 94.1, 91.7, 61.7, 43.8, 25.6, 20.7, 15.1, 14.5 ppm.

hrms: m/e calcd. for $C_{19}H_{18}O_7$: 358.1052; found: 358.1064 (76%); 343($C_{18}H_{15}O_7$)100; 328($C_{17}H_{12}O_7$)6; 325($C_{18}H_{13}O_6$)8; 299($C_{17}H_{15}O_5$)6.

The 8-Hydroxynaphthalic Anhydride trimethyl ether (100; 4,7,8-trimethoxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho [1,2-b] furan-5,6-dicarboxylic anhydride)

Formula (MW): $C_{21}H_{22}O_7$ (386).

ir ($CHCl_3$ cast): 2930, 1760, 1725, 1575, 1360, 1280, 1245, 1045, 1015 cm^{-1}.

^{1}H nmr ($CDCl_3$): 4.67(1H,q(7Hz),OCH); 4.14(3H,s,OCH_3); 4.13(3H,s,OCH_3); 3.92(3H,s,OCH_3); 2.88(3H,s,$ArCH_3$); 1.56(3H,s,CH_3); 1.52(3H,d(7Hz),CH_3); 1.32(3H,s,CH_3).

hrms: m/e calcd. for $C_{21}H_{22}O_7$: 386.1366; found: 386.1366 (97%); 371($C_{20}H_{19}O_7$)100; 353($C_{20}H_{17}O_6$)11; 343($C_{19}H_{19}O_6$)9; 327($C_{19}H_{19}O_5$)17.

3,6-Dihydroxy-4,5-dioxo-1,7,7,8-tetramethyl-4,5,7,8-tetrahydroacenaphtho [5,4-b] furan (101)

Formula (MW): $C_{18}H_{16}O_5$ (312).

Mp: 200-202°C (dichloromethane, red crystals).

ir ($CHCl_3$ cast); 3400, 2920, 1710, 1680, 1600, 1360, 1310, 1150, 1060, 920, 870 cm^{-1}.

^{1}H nmr ($CDCl_3$): 7.78(1H,bs(W1/2 = 16Hz),OH); 7.48(bs(W 1/2 = 16Hz), OH); 6.65(1H,q(1Hz),ArH); 4.67(1H,q(8Hz),OCH); 2.75(3H,d(1Hz),$ArCH_3$); 1.55(3H,s,CH_3); 1.48(3H,d(8Hz),CH_3); 1.29(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{18}H_{16}O_5$: 312.0998; found: 312.0995 (53%); 297($C_{17}H_{13}O_5$)100; 269($C_{16}H_{13}O_4$)43.

The Aromatic Hydroxy Acids (105-110)

As indicated in the discussion, small amounts of several compound mixtures were identified while still as mixtures. In this section the data for each separate compound identified will be presented as a unit.

p-Hydroxybenzoic acid (105)

Formula (MW): $C_7H_6O_3$ (138).

^{1}H nmr ($CDCl_3$/MeOH-$\underline{d}_4$): 7.94(2H,bd(8Hz),ArH); 6.84(2H,bd(8Hz),ArH) ppm.

hrms: m/e calcd. for $C_7H_6O_3$: 138.0317; found: 138.0317 (84%); 121($C_7H_5O_2$)100.

cims: 156 (M + NH_4^+).

The isolated mixture that includes compound 105 behaved as one compound when co-spotted on tlc with an authentic sample of *p*-hydroxybenzoic acid (silica gel; chloroform:methanol:acetic acid; 84:15:1).

Coumaric acid (106;E,3-(p-hydroxyphenyl)-2-propenoic acid)

Formula (MW): $C_9H_8O_3$ (164).

^{1}H nmr ($CDCl_3$/MeOH-$\underline{d}_4$): 7.64(1H,d(16Hz),=CH); 7.42(2H,d(8Hz),ArH); 6.84(2H,d(8Hz),ArH); 6.26(1H,d(16Hz),=CH) ppm.

hrms: m/e calcd. for $C_9H_8O_3$: 164.0473; found 164.0473 (71%); 163($C_9H_7O_3$)19; 147($C_9H_7O_2$)27.

cims: 182 (M + NH_4^+).

The isolated mixture that includes compound 106 behaved as one compound when co-spotted on tlc with an authentic sample of coumaric acid (silica gel; chloroform:methanol:acetic acid; 84:15:1).

3-(p-hydroxyphenyl) propanoic acid (107)

Formula (MW): $C_9H_{10}O_3$ (166).

^{1}H nmr ($CDCl_3$/MeOH-$\underline{d}_4$): 7.05(2H,d(8Hz),ArH); 6.77(2H,d(8Hz),ArH); 2.86(2H,bd(8Hz),CH_2); 2.58(2H,bd(8Hz),CH_2) ppm.

hrms: m/e calcd. for $C_9H_{10}O_3$: 166.0630; found: 166.0629 (25%); 107(C_7H_7O)86.

cims: 184 (M + NH_4^+).

Caffeic acid (108;E,3-(3',4'-dihydroxyphenyl)-2-propenoic acid)

Formula (MW): $C_9H_8O_4$ (180).

^{1}H nmr ($CDCl_3$/MeOH-$\underline{d}_4$): 7.58(1H,d(16Hz),=CH); 7.08(1H,d(2Hz),ArH); 6.96(1H,dd(8,2Hz),ArH); 6.84(1H,d(8Hz),ArH); 6.22(1H,d(16Hz),=CH) ppm.

hrms: m/e calcd. for $C_9H_8O_4$: 180.0422; found: 180.0421 (60%); 163($C_9H_7O_3$)13; 136($C_8H_8O_2$)49; 134($C_8H_6O_2$)16.

cims: 198 (M + NH_4^+).

Protocatechuic acid (109; 3,4-dihydroxybenzoic acid)

Formula (MW): $C_7H_6O_4$ (154).

^{1}H nmr ($CDCl_3$/MeOH-$\underline{d}_4$): 7.52(1H,dd(8,2Hz)ArH); 7.48(1H,d(2Hz),ArH); 6.87(1H,d(8Hz),ArH).

hrms: m/e calcd. for $C_7H_6O_4$: 154.0266; found: 154.0266 (100%); 137($C_7H_5O_3$)92; 109($C_6H_5O_2$)22.

cims: 172 (M + NH_4^+).

Esculetin (110; 6,7-dihydroxybenzo-2-pyrone)

Formula (MW): $C_9H_6O_4$ (178).

^{1}H nmr ($CDCl_3$/MeOH-$\underline{d}_4$): 7.60(1H,d(9Hz),=CH); 6.90(1H,s,ArH); 6.83(1H, s,ArH); 6.22(1H,d(9Hz),=CH).

cims: 196 (M + NH_4^+), 179 (M + H^+).

3,7-diacetoxy-5-hydroxy-4,6-dioxo-1,8,8,9-tetramethyl-5-(propan-2'-onyl)-5,6,8,9-tetrahydro-4H-phenaleno [1,2-b] furan (81)

Compound 78 (13 mg) was dissolved in dichloromethane (1.5 mL) and stirred at room temperature with pyridine (40 drops) and acetic anhydride (20 drops) for 11 hours. This reaction produced four main components (tlc). The major component, compound 81 was purified by ptlc (chloroform: methanol:acetic acid; 489:10:1)

Formula (MW): $C_{26}H_{26}O_9$ (482).

ir ($CHCl_3$ cast): 3440, 2970, 2925, 1780, 1755, 1705, 1615, 1420, 1365, 1335, 1235, 1185, 1035, 870, 755, 570, 510, 450 cm^{-1}.

uv (MeOH) λmax (ε): 215(32,000); 226(27,000, shoulder); 258(27,000); 340(7,700, shoulder); 366(8,500); 386(7,300, shoulder) nm.

^{1}H nmr ($CDCl_3$): 6.88(1H,q(1Hz),ArH); 4.68*(1H,q(7Hz),OCH); 3.02*(2H, ABq(15Hz),$COCH_2$); 2.85(3H,d(1Hz),$ArCH_3$); 2.38(3H,s,$COCH_3$); 2.28(3H, s,$COCH_3$); 2.25(3H,s,$COCH_3$); 1.52*(3H,s,CH_3); 1.48(3H,d,(7Hz),CH_3); 1.34(1H,s,OH); 1.26(3H,s,CH_3) ppm.

*Compound 81 is a diastereomeric pair (C-2) and the downfield signals attributable to equivalent hydrogens of that pair are reported as indicated.

^{13}C nmr ($CDCl_3$): 202.6, 194.5, 193.3, 169.0, 169.0, 166.1, 166.1, 152.5, 146.4*, 136.9, 122.8, 120.5*, 115.4, 114.6, 91.9, 83.5*, 49.3, 43.8*, 31.4, 26.0*, 23.8, 21.1*, 20.6*, 18.9, 14.7* ppm.

*Compound 81 is a diastereomeric pair (C-2) and the downfield signals attributable to equivalent carbons of that pair are reported as indicated.

hrms: m/e calcd. for $C_{26}H_{26}O_9$: 482.1577; found: 482.1582 (37%); 440($C_{24}H_{24}O_8$)68; 425($C_{23}H_{21}O_8$)100; 355($C_{20}H_{19}O_6$)22; 323($C_{19}H_{15}O_5$)22; 313($C_{18}H_{17}O_5$)39; 297($C_{17}H_{13}O_5$)16; 257($C_{14}H_9O_5$)9.

tlc: Rf 0.7 (chloroform:methanol:acetic acid; 184:10:1), yellow spot, orange fluorescence under uv light.

9-Methoxy naphthalic anhydride (82; 4-hydroxy-7-methoxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho [1,2-b] furan-5,6-dicarboxylic anhydride)

Ca. 40 mg of impure naphthalic anhydride (79) was dissolved in dichloromethane and stirred while slowly adding CH_2N_2 (ca. 0.3 M in ether). The reaction was monitored by tlc and concentrated after most of the starting material had reacted. The reaction mixture was purified by ptlc (chloroform:methanol:acetic acid; 989:10:1) providing pure compound 80 (10 mg) and compound 84 (2 mg).

Formula (MW): $C_{19}H_{18}O_6$ (342).

Mp: 234-236°C (dichloromethane/petroleum-ether, white crystals).

ir ($CHCl_3$ cast): 2920, 1760, 1650, 1615, 1590, 1470, 1355, 1310, 1220, 1120, 1010, 820 cm^{-1}.

uv (MeOH): 215(45,000); 256(63,000); 294(13,000, shoulder); 351(29,000); 364(26,000, shoulder); 382(22,000, shoulder) nm.

^1H nmr (CD_2Cl_2): 12.31(1H,s,OH); 6.97(1H,d(1Hz),ArH); 4.72(1H,q(7Hz), OCH); 4.12(3H,s,OCH_3); 2.89(3H,d(1Hz),$ArCH_3$); 1.54(3H,s,CH_3); 1.48(3H, d(7Hz),CH_3); 1.30(3H,s,CH_3) ppm.

^{13}C nmr (CD_2Cl_2): 167.1, 166.1, 165.3, 165.3, 157.0, 149.1, 137.1, 119.3, 112.6, 109.8, 101.1, 93.8, 92.4, 57.0, 43.9, 25.8, 24.3, 20.8, 14.7 ppm.

hrms: m/e calcd. for $C_{19}H_{18}O_6$: 342.1103; found: 342.1099 (42%); 327($C_{18}H_{15}O_6$)100; 309($C_{18}H_{13}O_5$)9; 303($C_{16}H_{15}O_6$)10; 283($C_{17}H_{15}O_4$)5; 267($C_{16}H_{11}O_4$)5.

cims: 702 (2M + NH_4^+), 360 (M + NH_4^+), 343 (M + H^+).

tlc: R_f 0.6 (chloroform:methanol:acetic acid; 989:10:1), light brown spot, uv visible.

4-Methoxy Naphthalic anhydride (84; 7-hydroxy-4-methoxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho [1,2-b] furan-5,6-dicarboxylic anhydride)

Formula (MW): $C_{19}H_{18}O_6$ (342).

ir ($CHCl_3$ cast): 2930, 1750, 1670, 1610, 1595, 1455, 1380, 1300, 1030, 865, 815, 790, 760 cm^{-1}.

uv (MeOH): 214(22,000); 230(inflection); 250(27,000); 273(inflection); 343(11,000); 373(12,000); 386(11,000, shoulder) nm.

^1H nmr (CD_2Cl_2): 6.94(1H,s,ArH); 4.68(1H,q(6Hz),OCH); 4.07(3H,s,OCH_3); 2.85(3H,s,$ArCH_3$); 1.52(3H,s,CH_3); 1.48(3H,d(6Hz),CH_3); 1.28(3H,s,CH_3) ppm.

hrms: m/e calcd. for $C_{19}H_{18}O_6$: 342.1103; found: 342.1101 (50%); 357($C_{20}H_{21}O_6$)29; 327($C_{18}H_{15}O_6$)100; 313($C_{17}H_{13}O_6$)7; 309($C_{18}H_{13}O_5$)11; 283($C_{17}H_{15}O_4$)16.

tlc: R_f 0.8 (chloroform:methanol:acetic acid; 989:10:1), light brown spot, uv visible.

Atrovenetinone ethanolate (87; 5-ethoxy-3,5,7-trihydroxy-4,6-dioxo-1,8,8,9-tetramethyl-5,6,8,9-tetrahydro-4H-phenaleno [1,2-b] furan)

Compound 85 (10 mg) was dissolved in 95% EtOH with heating and compound 87 crystallised on standing.

Formula (MW): $C_{21}H_{22}O_7$ (386).

Mp: colour fades *ca*. 115°C, darkens *ca*. 214°C and melts 223-26°C (EtOH, green crystals).

ir (CH_2Cl_2 cast): 3440, 2970, 2880, 1623, 1604, 1455, 1440, 1380, 1340, 1310, 1255, 1205, 1180,, 1169, 1155, 1045, 985, 930, 860, 815, 805, 798, 630, 575, 540, 520 cm^{-1}.

uv (EtOH) λmax (ε): 212(19,000, shoulder); 223(25,000); 252(30,000, inflection); 260(44,000); 270(7,900, inflection); 341(15,000), 360(9,400, inflection) nm.

hrms: m/e calcd. for $C_{21}H_{22}O_7$: 386.1365; found: 386.1361 (7%); 357($C_{19}H_{17}O_7$)20; 340($C_{19}H_{16}O_6$)28; 327($C_{18}H_{15}O_6$)12; 313($C_{18}H_{17}O_5$)33; 312($C_{18}H_{16}O_5$)30; 297($C_{17}H_{13}O_5$)100; 269($C_{16}H_{13}O_4$)42.

The Acetone Adduct of Ninhydrin (92; 2-hydroxy-1,3-dioxo-2-(2'-propanonyl) indane)

Ninhydrin hydrate (75 mg) was stirred in an acetone-acetic acid (2 mL, 99:1) solution for 60 hours. The reaction mixture was concentrated and the residue separated by ptlc (chloroform:methanol:acetic acid; 89:10:1) providing ninhydrin hydrate (29 mg) and compound 92 (36 mg).

Formula (MW): $C_{12}H_{10}O_4$ (218).

Mp: 139-140°C (chloroform, white crystals).

ir ($CHCl_3$ cast): 3320, 1745, 1708, 1585, 1425, 1365, 1330, 1275, 1180, 1080, 930, 740 cm^{-1}.

^1H nmr ($CDCl_3$): 7.94(4H,m,ArH); 3.76(1H,bs,OH); 3.31(2H,s,$COCH_2$); 2.12(3H,s,$COCH_3$) ppm.

hrms: m/e calcd. for $C_{12}H_{10}O_4$: 218.0580; found: 218.0577 (13%); 176($C_{10}H_8O_3$)100; 175($C_{10}H_7O_3$)16; 147($C_9H_7O_2$)19; 104(C_7H_4O)18; 76(C_6H_4)19.

tlc: R_f 0.6 (chloroform:methanol:acetic acid; 89:10:1), dark brown spot.

6,7-Dimethoxybenzo-2-pyrone (110a)

Compound 110 (ca. 1 mg) was dissolved in chloroform (1 mL) and 0.3 M diazomethane in ether was added at room temperature with stirring until no starting material remained by tlc. The reaction mixture was concentrated to give compound 110a in quantitative yield.

Formula (MW): $C_{11}H_{10}O_4$ (206).

^1H nmr (DMSO-d_6): 7.99(1H,d(10Hz),=CH); 7.29(1H,s,ArH); 7.11(1H,s, ArH); 6.32(1H,d(10Hz),=CH); 3.89(3H,s,OCH_3); 3.83(3H,s,OCH_3) ppm.

hrms: m/e calcd. for $C_{11}H_{10}O_4$: 206.0579; found: 206.0583 (100%); 191($C_{10}H_7O_4$)39; 178($C_{10}H_{10}O_3$)15; 163($C_9H_7O_3$)50; 120($C_7H_4O_2$)20.

Ethyl-3-(4-hydroxyphenyl)-2-(2-chloroacetamido) propanoate (41)

L-Tyrosine ethyl ester (10 g, 47.8 mmol.) was dissolved in chloroform (150 mL). Chloroacetyl chloride (8 g, 70.8 m mol.) was added dropwise with stirring at room temperature. After half of the acetyl chloride had been added a Na_2CO_3 solution (9 g in 50 mL H_2O)

was added at such a rate that addition of $ClCH_2COCl$ and Na_2CO_3 were completed simultaneously. The reaction was neutralised and extracted with chloroform.

Evapouration of the chloroform in vacuo gave a crystalline product, compound 41.

Formula (MW): $C_{13}H_{16}NO_4Cl$ (285).

^{1}H nmr ($CDCl_3$): 6.98(6H,m,ArH,NH); 4.80(1H,dt(10,ca.8Hz),CH); 4.20(2H, q(12Hz),OCH_2); 4.00(2H,s,$COCH_2Cl$); 3.25(2H,d(10Hz),CH_2); 1.25(3H,t(12Hz), CH_3) ppm.

hrms: m/e calcd. for $C_{13}H_{16}NO_4Cl$: 285.0768; found: 285.0775 (2%); 212($C_{10}H_{11}NO_2Cl$)10%; 192($C_{11}H_{12}O_3$)88; 147($C_9H_7O_2$)20; 120(C_8H_8O)17; 107(C_7H_7O)100; 77(C_6H_5)17.

3-(4'-Hydroxyphenyl) methylpiperazine-2,5-dione (42)

Compound 41 was dissolved in methanol in a pressure flask to which NH_4OH (70 mL) was added. The flask was cooled in an ice bath while gaseous ammonia was bubbled through the solution. The sealed flask was allowed to stand for 3 days. The reaction mixture was evapourated to near dryness in vacuo and the crystals which formed collected and recrystallised from water. Yield: 350 mg (3% from L-tryosine ethyl ester).

Formula (MW): $C_{11}H_{12}N_2O_3$ (220).

ir (nujol): 3320, 3180, 2960, 1665, 1610, 1460, 1375, 1330, 1220 cm^{-1}

^{1}H nmr (DMSO-d_6): 9.24(1H,bs(W1/2 = 6Hz),ArOH); 8.02(1H,bd(3Hz),NH); 7.79(1H,bd(2Hz),NH); 6.94(2H,d(8Hz),ArH); 6.64(2H,d(8Hz),ArH); 3.96(1H, m(W1/2 = 8Hz),CH); 3.33(1H,dd(17,3Hz),HCH); 2.99(1H,dd(14,4Hz),HCH); 2.73(1H,dd(14,5Hz),HCH); 2.71(1H,d(17Hz),HCH) ppm.

hrms: m/e calcd. for $C_{11}H_{12}N_2O_3$: 220.0849; found: 220.0849 (9%);

114($C_4H_6N_2O_2$)26; 107(C_7H_7O)100; 77(C_6H_5)10.

(S)N,N-dimethyl-3-(4'-methoxyphenyl) methylpiperazine-2,5-dione (27)

Compound 42 (213 mg, 0.97 m mol.) was dissolved in warm dry tetrahydrofuran (50 mL) and the minimum amount of DMSO required to form a homogeneous solution. NaH (244 mg (57% oil dispersion), 5.8 m mol.) was added and the reaction mixture was allowed to stir at room temperature for two hours. Excess dimethylsulphate (1 mL) was added dropwise and the reaction mixture was allowed to stir a further 2 hours. The mixture was neutralised (1*N* HCl), reduced in volume *in vacuo* and extracted several times with ethyl acetate. The combined ethyl acetate extracts were washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evapourated *in vacuo* providing 173 mg (68%) of compound 27 recovered as a white, crystalline solid.

Formula (MW): $C_{14}H_{18}N_2O_3$ (262).

Mp: 133-135°C (dichloromethane, white crystals).

cd (*c* 0.0084, MeOH) 26°: $[\theta]_{224}$ - 9570 , $[\theta]_{210}$ 0 .

ir ($CHCl_3$ cast): 2920, 1680, 1610, 1510, 1495, 1460, 1400, 1335, 1245, 1175 cm^{-1}.

^1H nmr ($CDCl_3$): 6.97(2H,d(8Hz),ArH); 6.82(2H,d(8Hz),ArH); 4.15(1H, t(4Hz),CH); 3.79(3H,s,OCH_3); 3.33(1H,d(17Hz),$\underline{H}CH$); 3.22(1H,dd(14,3.5Hz), $\underline{H}CH$); 3.06(3H,s,NCH_3); 3.04(1H,dd(14,4Hz),$\underline{H}CH$); 2.74(3H,s,NCH_3); 2.38(1H,d(17Hz),$\underline{H}CH$) ppm.

^{13}C nmr ($CDCl_3$): 165.8, 164.2, C=O; 159.4, ArC; 130.8, 2 x ArCH; 126.4, ArC; 114.0, 2 x ArH; 63.6, CH; 55.2, OCH_3; 50.9, 36.2, CH_2; 30.0, 32.2, NCH_3 ppm.

hrms: m/e calcd. for $C_{14}H_{18}N_2O_3$: 262.1317; found 262.1315 (5%); 121(C_8H_9O)100.

tlc: R_f 0.2 (chloroform:methanol:acetic acid; 89:10:1), light brown spot.

(S)Methyl-2-amino-3-phenylpropanoate (45)

L-Phenylalanine (5 g) was suspended in ether and diazomethane in ether (0.3 M) was added over 60 hours until the solution was homogeneous and remained a yellow colour. The reaction mixture was concentrated *in vacuo* to give a quantitative yield of compound 45.

Formula (MW): $C_{10}H_{13}NO_2$ (179).

ir ($CHCl_3$ cast): 3380, 3320, 2950, 1740, 1605, 1445, 1200, 1175, 750, 700 cm^{-1}.

^1H nmr ($CDCl_3$): 7.20(5H,m,ArH); 3.7(1H,m,CH); 3.68(3H,s,OCH_3); 3.08(1H, dd(14,5.5Hz)HCH); 2.83(1H,dd(14,8Hz),HCH)ppm.

hrms: m/e calcd. for $C_{10}H_{13}NO_2$: 179.0946; found: 179.0952 (1%); 120($C_8H_{10}N$)86; 102($C_4H_8NO_2$)39; 91(C_7H_7)33; 88($C_3H_6NO_2$)100.

(S)2-Amino-3-phenylpropanol (46)

Compound 45 (4.4 g, 0.029 mol) in ether (80 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (3 g, 0.079 mol) in anhydrous ether (60 mL) . The reaction mixture was allowed to stir for a further hour then treated consecutively with H_2O (3 mL), 10% NaOH (4.5 mL), and H_2O (8 mL). The reaction mixture was filtered and extracted with ether. The ether extract was dried, filtered and concentrated to yield crude compound 46 which was purified by silica gel chromatography.

Formula (MW): $C_9H_{13}NO$ (151).

Mp: 86-88°C (ether), 85-86°C (lit.).

ir ($CHCl_3$ cast): 3360, 3300, 2920, 2880, 1580, 1495, 1457, 1340, 1065, 755, 700 cm^{-1}.

^1H nmr ($CDCl_3$): 7.20(5H,m,ArH); 3.61(1H,dd(10,3.5Hz),HCH); 3.36(1H, dd(10,7Hz),HCH); 3.08(1H,bs(W1/2 = 18Hz),CH); 2.78(1H,dd(12,5Hz),HCH);

2.48(1H,dd(12,8Hz),HCH); 2.46(ca.3H,bs) ppm.

hrms: no parent ion; 120($C_8H_{10}N$)33; 91(C_7H_7)18; 60(C_2H_6NO)100.

(S)N-(1-hydroxymethyl-2-phenylethyl) benzamide (43)

Compound 46 (300 mg, 2.0 m mol) was dissolved in pyridine (2 mL) and benzoyl chloride (540 mg, 3.8 m mol.) was added and the reaction mixture was allowed to stand overnight. The pyridine was removed in vacuo and the reaction mixture poured onto ice-water providing a crystalline product. The crystals were dissolved in ethanol (9 mL), NaOH (78 mg) was added and the reaction mixture was heated under reflux for 1 hour. The ethanol was removed in vacuo and the solid product was dissolved in chloroform, washed (H_2O) and dried (sat. NaCl, Na_2SO_4). Excess chloroform was removed in vacuo giving compound 43 which was recrystallised from ethanol.

The spectral and physical properties (ir, nmr, mp and $[\alpha]_D$) of compound 43 are identical in all respects with the reported values[58-60]. The compound has previously been isolated from P. brevi-compactum.

2-(1,2-Dioxopropyl) aminobenzamide (57)

$FeSO_4.7H_2O$ (110 g, 0.4 mol, in 110 mL hot H_2O) was added to a vigorously stirred solution of 2-nitrobenzamide (5 g, 0.03 mol) in hot H_2O (60 mL). Concentrated ammonia (NH_4OH) was added to basify the mixture and the mixture was allowed to boil for 5 minutes. The hot solution was filtered, the filtrate reduced in volume in vacuo, then cooled. Crystals of 2-aminobenzamide were collected by filtration. Yield 1 g (25%).

Pyruvyl chloride was synthesised in the following manner. Dichloromethyl methyl ether (13.1 g, 0.114 mol) was added dropwise with stirring to pyruvic acid (10 g, 0.114 mol) at room temperature. After the addition was complete the reaction was warmed to 50° for 30 minutes and the product collected by distillation (ca. 1 g, bp. 53° at 126 torr).

The synthesis of compound 57 was completed by the condensation of 2-aminobenzamide and pyruvyl chloride. 2-aminobenzamide (0.53 g, 3.9 m mol) was dissolved in ethyl acetate (10 mL). Na_2CO_3 (0.62 g, 5.8 m mol) was added to the solution which was then cooled to 0°C. Pyruvyl chloride (0.46 g, 4.3 m mol) was added dropwise with stirring and the reaction allowed to warm to room temperature. The reaction was quenched (20 mL H_2O), diluted (30 mL EtOAc), washed (2 x 20 mL sat. $NaHCO_3$, 20 mL H_2O) dried (anhydrous Na_2SO_4), filtered and evapourated in vacuo. The residue was recrystallised from methanol/H_2O to provide 0.2 g of a white crystalline compound.

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